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REVERSIBLE AZIDE INHIBITION OF WATER INFLUX INTO INDIVIDUAL RADISH ROOT-HAIR CELLS

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FOUR FIGURES

INTRODUCTION

The active reabsorption of water by the kidney tubule cells is a well known example of the transfer of water against an osmotic gradient, but the nature of the mechanism which gears the functional process to metabolism is unknown. Perhaps this mechanism is of universal occurrence and further insight into its nature may be provided by studying simpler systems. The present study is new in that the effects of an oxidative inhibitor (sodium azide) on water transfer were determined when the inhibitor was applied to a localized portion of a single cellular projection, the root-hair cell. The experiments not only demonstrate reversible azide inhibition of water influx through the hair-cell surface, but they also demonstrate the successful application of a technique to study the effects of various drugs on water influx through unit surface area of a portion of an individual cell when it is exposed to drug action.

PROCEDURE

The technique described by Rosene ('43, '50) was modified for the present study. Previous work (Rosene and Bartlett, '50) demonstrated the feasibility of carrying out measurements over periods of three hours or longer on a single root-

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hair. Since most of the experiments in the present investigation consisted of three 60-minute periods between which micropotometers were removed from the chamber, emptied, flushed, and refilled (a "changeover") with different solutions to test their effect on the rate of water influx on the same root-hair, it was mandatory that operations be rapid so that opening of the chamber would not affect environmental conditions appreciably within the chamber. The total time the chamber was open during a "changeover" was less than 20 seconds — a period which had little or no effect on humidity conditions within the chamber.

Temperature in the chamber was consistent to plus or minus .2°C. No correlation was found between the rate of water absorption and this small variation in temperature during an experiment. Unless otherwise indicated all dilutions of pure sodium azide and all control solutions were made with 25% aerated Hoagland's solution and adjusted to pH 6.2. In all experiments the root-hairs of *Raphanus sativus* were inserted one ocular division or to a length so that 8,666 μ^2 of the surface area of the root-hair was immersed within the micropotometer. The length of root-hairs selected for experimentation were between 805 μ and 960 μ. All experimental root-hairs were grown in the chamber and were in a region from 6 to 9 mm from the root apex. The experiments were made on root-hairs of growing radish seedlings with one exception as noted in a following section which describes experiments on root-hairs of excised roots.

RESULTS

Controls. Experiments were run to see what effect "changeover" would have on the rate of water influx. Hoagland's solution was used in all three periods, and as shown by a typical curve in figure 1A; "changeovers" did not interrupt significantly the rate of water influx; hence, it was found feasible to run comparatively long experiments. Vertical arrows represent "changeovers." Each symbol represents

the average velocity of water influx during a 10 minute period. Average rates of water influx in cubic microns per square micron of root-hair surface per minute for the three periods were 1.18, 1.19, and 1.24 respectively.

Azide series. Variable results were obtained with exposures to .0025 M, .005 M, or .01 M azide. Figure 1B represents a typical curve obtained when a root-hair was exposed

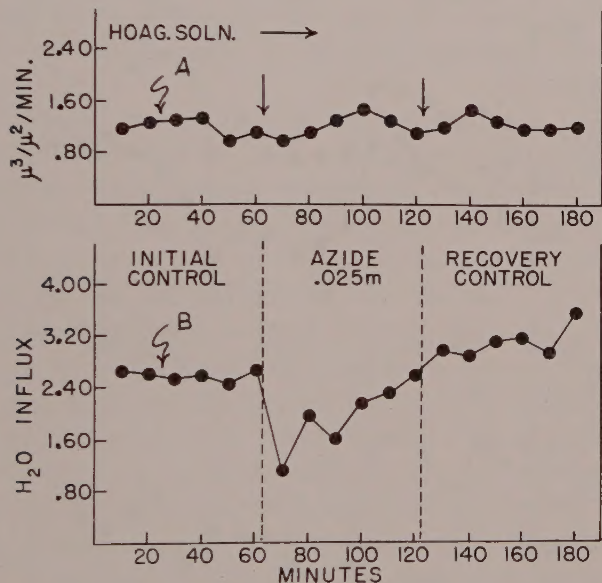

Fig. 1 Curve A. Time course of water influx per unit surface area of root-hair cell exposed to 25% Hoagland solution. Arrows indicate change of solution. Temp. 29.1°C. Curve B. Effect of .025 M sodium azide on water influx. Temp. 29.7°C.

to .025 M azide. The curve shows a distinctive decrease in the rate of water influx during the initial exposure to azide. Subsequently in the presence of azide a partial recovery was manifested. Curves A, B, and C of figure 2 show the characteristic drop of water influx to the relatively low inhibitory level manifested at concentrations of .04 M to .10 M azide. Complete reversible recovery occurred at concentrations of .04 M azide. Curve A óf figure 2 shows the rates in the re-

covery control period to be significantly greater than the rates in the initial control period. This is referred to as the "overshooting" phenomenon which was found in all the experiments but one (fig. 2B) when root-hairs of intact roots were exposed to .04 M azide. The pattern of recovery following exposure to concentrations of .075 or .1 M azide was

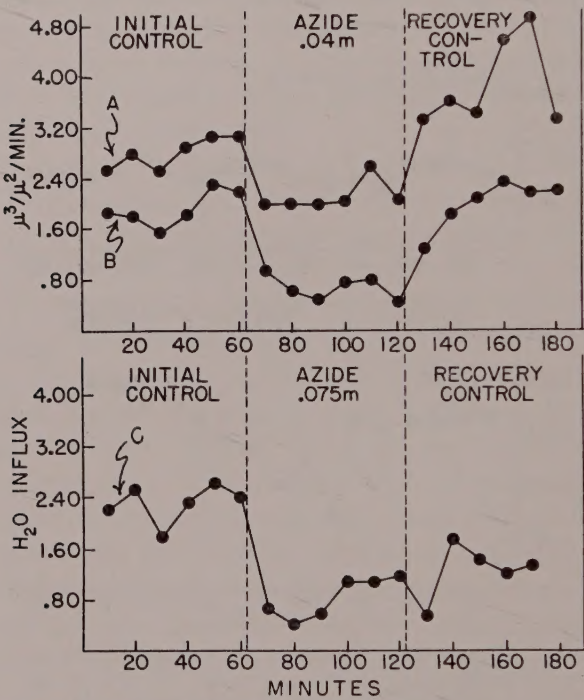

Fig. 2 Curves A and B. Reversible azide inhibition of water influx. .04 M azide. Temp. 27°C. Curve C. Effect of .075 M azide on water influx. Temp. 28.7°C.

variable exhibiting: (a) complete recovery as in the .04 M azide series with or without "overshoot," (b) incomplete recovery as shown by curve 2C, or (c) no recovery. At these higher concentrations root-hairs frequently collapsed during exposure of a small area to azide.

Excised roots. Rosene ('41) found that the osmotic pressures of solutions required to stop water uptake of excised

onion roots were lower than those of intact roots due perhaps to the fact that in excision, any ''shoot-pull'' mechanism was eliminated. With a piece of razor blade, the radish seed and/or young shoot was excised at the base of the radicle without disturbing atmospheric conditions around the seedling and then two hours were allowed before measurements were made. Since .04 M azide was found to be a concentration which always showed inhibition, it was thought that this concentration could be used to compare any differences in the degree of inhibition which might be ascribed to a ''shoot-pull'' mechanism.

The largest per cent of azide inhibition of water influx (90% as compared to 51–79% with intact roots) was observed under these experimental conditions (fig. 3A). Other than this observation, the general appearance of the curve obtained is similar to curves of the .04 M azide series (Cf. figs. 2A, 2B, 3A).

Distilled water solutions. Experiments in which root-hairs were exposed to .04 M azide in distilled water solutions showed curves (fig. 3, A and B) similar to those of figure 2, A and B thus demonstrating that whatever the effects of azide, they were evidently due to azide and not to some complex formed between azide and elements found in Hoagland's solution.

When root-hairs were first exposed for 60 minutes to dilute aerated Hoagland's solution and then for 60 minutes to aerated distilled water, there was no discernible difference in the rates of water influx for the two periods (fig. 3C). Whatever osmotic or salt accumulation forces may be operative in root-hairs when using dilute Hoagland's solution, they were not evident in these experiments.

pH series. Stenlid ('48) and Armstrong and Fisher ('40) stressed the importance of pH in azide studies, maintaining that the concentration of the undissociated form of hydrazoic acid determines the degree of inhibition of the process being measured. Stannard ('39), however, did not observe any pH effects of azide on the inhibition of oxygen consumption in active frog muscle. The fact that complete azide inhibition

in vivo was not attained in his studies emphasizes that (a) azide may behave differently *in vivo,* (b) possibly other equally important oxidative processes are involved or, (c) perhaps the selective permeability of some membranes to certain pH values of azide solutions determines the degree

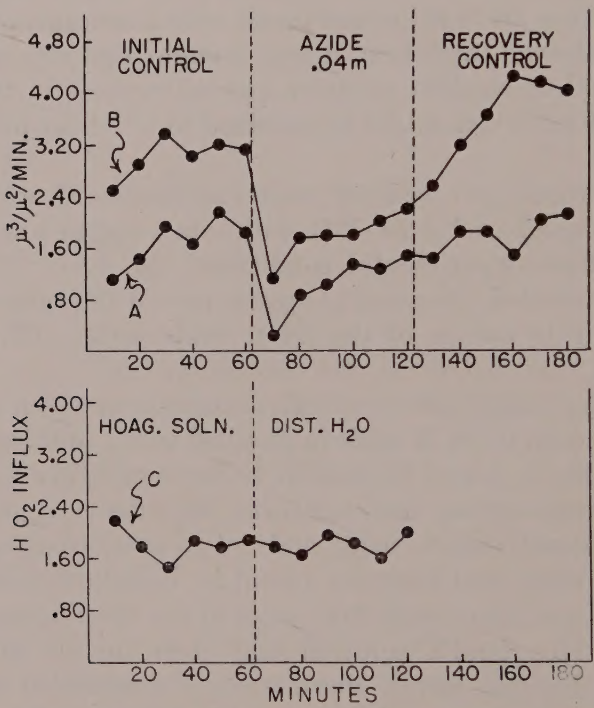

Fig. 3 Curve A. Effect of .04 M azide on water influx into a root-hair of an excised root. Temp. 29°C. Curve B. Effect of .04 M azide solution made with distilled water. Temp. 30.3°C. Curve C. Comparative influx of 25% Hoagland solution and of distilled water. Temp. 24.4°C.

of inhibition. Figure 4A presents the results of two experiments performed with the same azide concentration (.04 M) as the experiment represented by figure 2, A and B except that instead of using solutions adjusted to pH 6.2, in one instance the solutions were adjusted to pH 5.0, in the other to 7.2. The curves do not show a significant difference be-

tween themselves or when compared to curves A and B of figure 2. Within this comparatively narrow pH range of .04 M azide, it appears that a particular hydrogen ion concentration was not indicative of a particular percentage of azide inhibition or of overshooting during recovery.

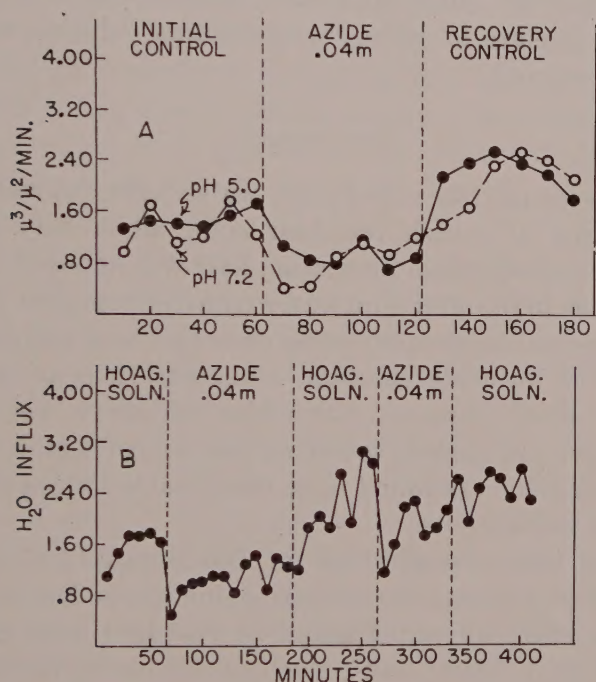

Fig. 4 Upper curves. Comparative azide inhibition of water influx at different pH values. Temp. 29.8°C. Bottom curve. Comparative effect of intermittent exposure to .04 M azide. Temp. 24°C.

Multiple test period. Figure 4B represents an experiment designed to determine if (a) longer exposure or (b) repeated exposure to .04 M azide would increase inhibition or cause the root-hair to collapse. While it is evident from the graph that during the test periods, rates always remained at an inhibitory level as compared to the immediately preceding rates when in dilute Hoagland's solution, recovery and marked "overshooting" occurred. It is noted that though the root-

hair was still absorbing water at a high rate during the time it was exhibiting "overshooting," when azide was again applied, it dropped to a new inhibitory level. By calculating inhibition from the last rate shown before azide was added, the percentages of inhibition of the two test periods were similar, however (67% and 62% respectively). Following repeated exposure to azide, "overshooting" during recovery was not observed.

DISCUSSION

The effects on the velocity of water influx through unit surface area of radish root-hair cells, when only a small portion of an individual projecting hair was exposed to azide, were similar to the effects on water transfer reported (Rosene, '47) for excised onion roots when each root was almost totally immersed in an azide solution. Concentrations of azide required to show maximum reversbile inhibition were in the same range. An azide-insensitive fraction of water transfer in root-hair cells was found to be comparable to that observed in excised onion roots.

The fact that concentrations of .005 M and .01 M azide reduced water transfer in excised onion roots but showed a negligible effect on water influx of root-hair cells might be explained by a "shoot-pull" mechanism present in the radish seedling which may have masked a comparatively small reduction at these lower concentrations. Evidence in favor of this explanation is based upon the experiments with .04 M azide (fig. 3, A and B) which showed a greater percentage inhibition in the absence of the hypocotyl or "shoot-pull" and upon experiments of the effect of water absorption by intact onion roots performed in this laboratory (unpublished results). As mentioned in the study on onion roots, further work is needed to elucidate the "overshooting" phenomena which occurs during recovery from azide inhibition at certain concentrations.

The reduction in water influx through unit hair-cell surface might be attributed, at least in part, to an increased osmotic pressure of the azide solution. The fact that at the higher concentrations of azide greater increases in percentage reduction of water absorption did not occur is against an explanation based entirely on osmotic effects. Although no data are available of the effective osmotic pressure, which will reduce water transfer in the radish root-hair to the level exhibited at higher concentrations of azide, the osmotic pressure of the strongest azide solution used was below that which will stop water influx into the root-hair cells of onion seedlings as found in this laboratory.

It is believed that the decreased velocity of water influx into the radish root-hair cell may be attributed chiefly to the effect of azide on metabolism. In support of this conclusion is the well known action of azide as an oxidase inhibitor and the fact that percentage inhibition of water transfer and percentage inhibition of oxygen consumption by azide in onion roots were found (Rosene, '47) to follow parallel courses. Additional support of the conclusion that cellular oxidations are a direct determinant in the rates of water transfer is based upon the experiments which demonstrated that water absorption by radish root-hair cells was inhibited by anoxia (Rosene and Bartlett, '50) under comparable conditions.

Lundegardh ('49) and Hackett and Thiman ('50) have also reported azide inhibition of water uptake by tissues. In their work and the work on onion roots, an extended surface area which included many cells was exposed to azide. But in the present experiments, the area of exposure was limited to a portion of cellular surface. This fact demonstrates that the reversible inhibitory effects of azide on water influx were probably due to effects on cellular oxidations of the hair-cell *per se*. In the investigation on anoxia (Rosene and Bartlett, '50) the seedling itself was exposed to anaerobic conditions during the anoxic period, hence the experiments did not show

directly whether or not the decrease in water influx of the hair cell was the result of an anoxic condition of the hair and/or the result of other conditions.

SUMMARY

Rosene's microtechnique was modified to determine the effect of azide on water absorption by a localized region of an individual root-hair cell when the region was exposed to azide in aerated Hoagland's solution, diluted to one-fourth strength at pH 6.2. The average influx in cubic micra of water passing through square micron of exposed hair surface per minute was determined during three successive time periods, an initial control, an azide test period, and a recovery control. Concentrations of .0025 M, .005 M, and .01 M azide showed negligible or no effects. Perhaps a "shoot-pull" mechanism in the growing seedling masked effects produced at these concentrations. A characteristic reduction of water influx to an inhibitory level was found at concentrations of .04 M, .075 M, and .1 M azide. Maximum reversible inhibition occurred at .04 M azide with the per cent inhibitions ranging from 51 to 79%. Irreversible inhibition and injury effects were common at concentrations of .075 M and .1 M azide. The greater per cent (90%) azide inhibition of water transfer by a root-hair was observed when using an excised root which was believed to be due to the fact that in excision the "shoot-pull" mechanism was removed. It was shown that the inhibiting action of azide was due to azide alone and not to some complex formed between azide and elements found in Hoagland's solution. There was an absence of effects of hydrogen ion concentration at pH 5.0, 6.2, and 7.2 during inhibition by .04 M azide. The data show that decrease in water transfer was due to alteration in forces largely localized in the root hair. The inhibiting action of azide on water influx in radish root-hairs supports the view that at least part of the water transfer process is associated with cellular oxidations.

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CONTROL OF CELL DIVISION IN BACTERIAL CULTURES [1]

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FOUR FIGURES

The cessation of growth in bacterial cultures has been attributed to the depletion of some nutrient factor or factors essential to the anabolic processes of the cells (e.g., Barnes, '31; Jordan and Jacobs, '44; Lockhart and Powelson, '53a). By studying cultures in which total growth was limited by a single nutrient we have found that reproduction of the cells ceases at a time when that substrate is still available. For a short period thereafter the cells continue to utilize the substrate. During this time a spectrum of cellular changes develops that is similar to one manifested in the late lag and early logarithmic phases of growth. It was also observed that at the end of the growth period the level of residual glucose varied characteristically for each of the bacterial strains tested under these conditions, with glucose as the limiting substrate.

METHODS

The test organism *(Escherichia coli,* strain 61, except where noted otherwise in the text) was grown in a synthetic

[1] A portion of the thesis presented by the senior author to the faculty of Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A preliminary report of these data was made before the 53rd annual meeting of the Society of American Bacteriologists at San Francisco in August, 1953 (Lockhart and Powelson, '53b).

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medium containing 5.4 gm of KH_2PO_4, 1.2 gm of $(NH_4)_2SO_4$, 0.4 gm of $MgSO_4 \cdot 7H_2O$ and 3.0 gm of glucose per liter of glass-distilled water, pH 7.1 before autoclaving. The concentrations of glucose and $(NH_4)_2SO_4$ were varied in some experiments, as indicated in the text. Cultures were incubated at 37°C. either in a series of optically calibrated culture tubes through which sterile air was bubbled or in a two-liter vessel, aerated by means of a fritted-glass sparger and bearing an attachment by which samples could be removed at intervals without risk of contamination.

Optical densities of growth were determined with a Coleman M9 nepho-colorimeter at 655 mμ. Total cell counts were made with a hemacytometer, viable counts by the usual plate-count technique on nutrient agar. Sensitivity to phenol was determined by mixing 1.0 ml of 1.0% phenol with 1.0 ml of culture and making a plate count of the cells surviving after three minutes at 37°C. Per cent killing was calculated by comparison of this count with that obtained from the untreated culture.

Ribose and desoxyribose nucleic acids (RNA and DNA) were extracted with perchloric acid by the method of Ogur and Rosen ('50) and measured with the Beckman DU spectrophotometer at 260 mμ. Quantities of nucleic acid were expressed in arbitrary units; 1 mg of commercial RNA (Nutritional Biochemicals) was found to be equivalent to 25 arbitrary units.

Respiratory activity of cells was determined by the method of Kopper ('52). The amount of 2,3,5,-triphenyltetrazolium chloride reduced in 15 minutes by 2 to 9 billion cells in the presence of glucose was measured optically at 430 mμ. Residual substrates in culture fluids were determined after removal of the cells by centrifugation. Glucose was determined by an anthrone method, ammonia nitrogen by a phenol-hypochlorite method (Niss, '54). Detailed outlines of these analytical methods are given by Lockhart ('54).

RESULTS

The data presented here summarize results obtained with aerated cultures in a medium containing 3.0 gm of glucose and 1.2 gm of ammonium sulfate per liter. Experiments showed that under these conditions, the total bacterial population is limited by the supply of glucose. In cultures of *E. coli,* strain 61, the pH of the medium changes from 7.1 at

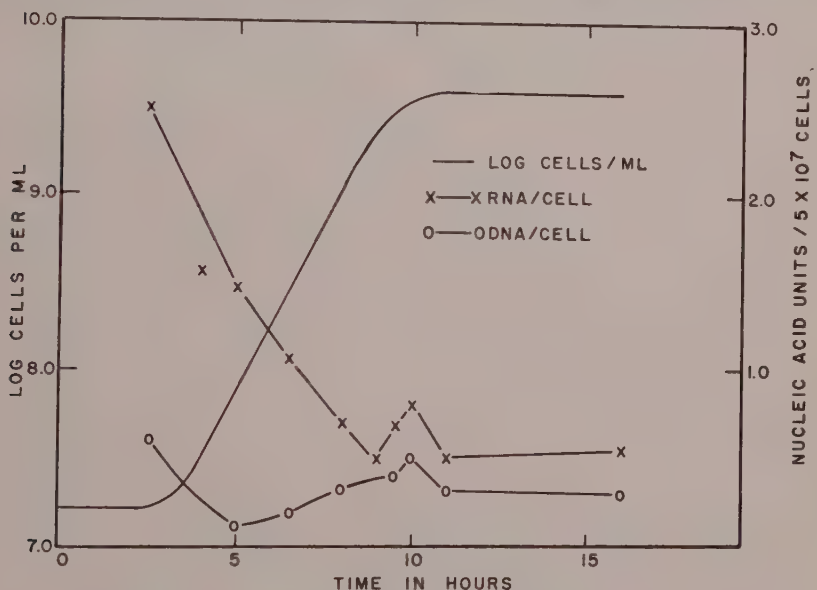

Fig. 1 Variation in nucleic acid content of cells during growth of *E. coli.* A composite of data from several experiments. Log cells/ml = logarithm of total cell count per milliliter of culture. RNA and DNA = amount of nucleic acid (in arbitrary units) per 5×10^7 cells.

inoculation to 6.8 at the onset of the stationary phase to about 6.5 at the time that observations are discontinued. The changes in composition of the medium which are responsible for these pH changes have not been investigated.

Figure 1 illustrates the variation in the nucleic acid content of the cells during cultural growth. As has been observed by many workers (e.g., Morse and Carter, '49), the concentrations of RNA and DNA per cell are high just before

logarithmic growth, then decrease with time. During the transition from logarithmic to stationary growth phases, when reproduction no longer proceeds at an exponential rate, there occurs a temporary but significant increase of nucleic acids in the cells. The increase of RNA is relatively greater than that of DNA so that the ratio of RNA to DNA also rises at this time. Since the method of perchloric acid extraction is most likely to err by indicating high values of

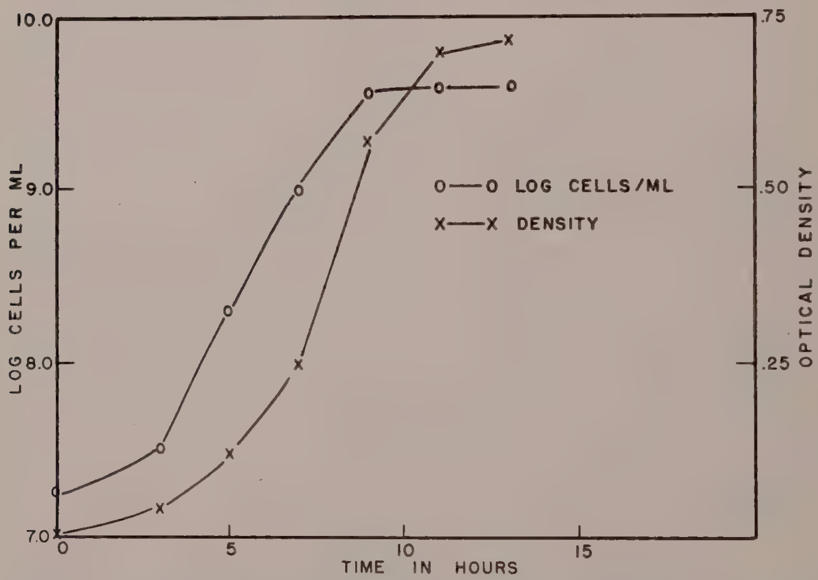

Fig. 2 Increase in optical density during stationary phase in a culture of *E. coli*. Log cells/ml = logarithm of total cell count per milliliter of culture. Density = optical density of culture at 655 mμ.

DNA (because all the RNA may not be removed in the first fraction), this altered ratio is unlikely to be an artifact. As indicated in figure 1, repeated experiments show that the increase of DNA apparently begins before that of RNA.

There is also an increase in cell size. Figure 2 shows that optical density in these cultures becomes considerably greater during the period at the end of logarithmic cell division though the number of cells is increasing only slightly or not

at all. Studies with diluted suspensions of cells and other insoluble particles have shown that the relationship between optical density and number of particles in suspension is nearly linear for our instrument throughout the density range encountered in these experiments. A statistical test showed that the increase in the optical density during this period

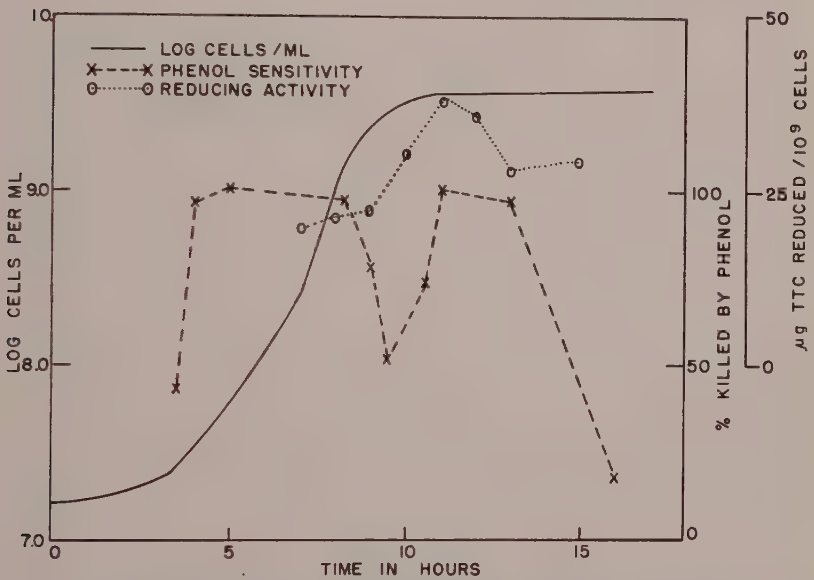

Fig. 3 Variations in phenol sensitivity and rate of respiration during growth of *E. coli*. A composite of data from several experiments. Log cells/ml = logarithm of total cell count per milliliter of culture. Phenol sensitivity = per cent of cells killed by exposure to 0.5% phenol for three minutes. Reducing activity = µg of 2,3,5-triphenyltetrazolium chloride reduced in 15 minutes per 10^9 cells.

was significant. Direct microscopic observations have confirmed this indication that the average cell size increases in the early part of the stationary phase of growth.

Physiological changes in the cells were also observed at this stage of growth. Figure 3 shows the variation in phenol sensitivity and in respiratory activity. As noted also by other workers (e.g., Sherman and Albus, '23), sensitivity to phenol becomes high as growth begins and then decreases sharply near the end of the period of active reproduction.

However, we find a second temporary peak in sensitivity during the period when the changes already mentioned occur. There is also a temporary increase in respiratory activity, as measured by the amount of tetrazolium chloride reduced per cell. Kopper ('52) has shown that a high capacity for tetrazolium reduction is characteristic also of cells from "young" cultures.

This pattern has been observed under a variety of environmental conditions (Lockhart, '54). Similar results were obtained in aerated cultures with glucose limiting the total extent of growth (at three different levels of initial glucose concentration); in aerated cultures with nitrogen limiting (at two levels of initial ammonium sulfate concentration); and in unaerated cultures in which both these substrates were in excess and growth was apparently limited by lack of oxygen. *Aerobacter aerogenes,* grown with citrate as the limiting carbon source in one experiment, underwent similar changes during the early part of the stationary phase of the culture.

It is evident that synthetic processes and cellular growth continue in these cultures for a time after cell division ceases. Determinations of residual glucose and nitrogen in culture fluids have shown that substrates are used during this period. Figure 4 illustrates the utilization of both glucose and nitrogen in an aerated culture containing 3.0 gm of glucose and 0.25 gm of ammonium sulfate per liter of medium. Under these conditions nitrogen is limiting the extent of growth. It will be noted that the stationary phase is reached while there is still nutrient available, and the cells continue to use both substrates for a time after reproduction has ceased. At the end of this period nearly all the nitrogen has been used, while the glucose concentration remains at a higher level than that which would limit growth if nitrogen were in excess.

Similar results are obtained when glucose is limiting (table 1). The end of exponential reproduction occurs while the limiting substrate is still available, and the cells continue

to utilize the glucose for a time thereafter. Although the last column of table 1 shows an apparent decrease in the rate of substrate utilization at the onset of the stationary

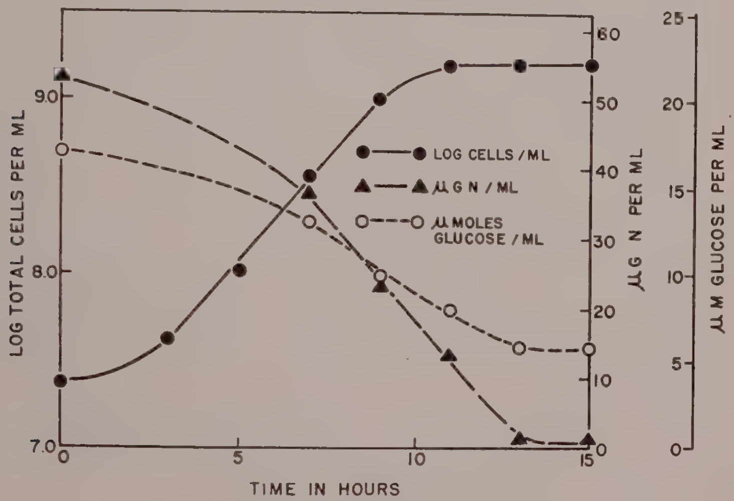

Fig. 4 Utilization of substrates in an aerated culture of *E. coli.* Synthetic medium; total population limited by the availability of nitrogen.

TABLE 1

Utilization of glucose in an aerated culture of E. coli

INCUBATION TIME	TOTAL CELLS/ML	RESIDUAL GLUCOSE	CALCULATED RATE OF UTILIZATION OF GLUCOSE [1]
hours	$\times 10^7$	*µmoles/ml*	*µmoles/10⁹ cells/hour*
0	1.5	23.0	..
3	1.5	..	..
5	2.8	..	..
7	14.3	22.5	..
8	26.3	21.9	2.99
9	55.0	20.6	3.34
10	118.0	15.8	5.77
10½	194.0	11.1	6.25
11	174.0	7.5	3.91
11½	191.0	2.5	5.52
12	190.0	..	..
12½	200.0	2.5	0.00

Initial concentration of $(NH_4)_2SO_4$ in medium = 1.2 gm per liter.

[1] The formula used in calculation is given by Rahn ('38).

phase, the data obtained so far are insufficient to show whether the observed changes in rate are significant.

We have found that in this medium exponential cell division ceases quite abruptly; there is apparently no transitional period during which rate of reproduction gradually decreases before the onset of the stationary growth phase (Lockhart, '54). Apparently, the concentration of the substrate present at this time is insufficient to support further

TABLE 2

Residual glucose in aerated cultures of various organisms at the end of growth

STRAIN	OPTICAL DENSITY	TOTAL POPULATION	RESIDUAL GLUCOSE	
		cells/ml × 10^9	*µmoles/ml*	*µmoles/10^9 cells*
A. aerogenes Strain M	.43	1.81	0.31	0.17
	.42	1.50	0.28	0.19
E. coli Strain M	.53	3.27	0.81	0.25
E. coli Strain Gr	.50	2.45	0.83	0.34
	.50	2.45	0.78	0.32
E. coli Strain H52	.42	2.07	1.11	0.54
	.43	1.50	0.97	0.65
E. coli Strain 61	.60	1.90	2.50	1.31
	.57	2.00	2.50	1.25

Values recorded are from duplicate cultures.

cell division. The minimum substrate concentration required for reproduction seems to vary with individual bacterial strains. In one set of experiments, the concentration of glucose in a culture of *E. coli*, strain 61, at 10½ hours when reproduction ended was 5.7 µmoles/billion cells, by calculation from table 1. In a similar culture of *Aerobacter aerogenes*, strain M, cell division ceased when the glucose concentration was 1.7 µmoles/billion cells.

The fact that the amount of residual substrate apparently reaches a constant level in the late stationary phase (fig. 4; table 1) prompted a comparison of several bacterial strains on this basis. Various strains were grown in aerated cultures with glucose limiting, and the end point of growth (including the post-logarithmic period of growth without reproduction) was taken as the time when no further increase was observed in optical density. Total cell counts and analyses for residual glucose made at this point yielded the data shown in table 2. The concentration of residual glucose which appeared to limit growth varied with the different strains of organisms. These results suggest an explanation for the phenomenon of "staling" among various strains and species of bacteria. It is significant that these values of substrate concentrations which limit growth of the test strains can be correlated with the staling capacities of the same strains, i.e., those strains capable of staling media against the others (cf., Lockhart and Powelson, '53a) continue to grow until a concentration of glucose is reached which is much lower than concentrations limiting growth of the other strains.

DISCUSSION

It is obvious that a culture of bacteria may stop growing for any of a number of reasons depending upon the organism, the medium and the physical environment. The accumulation of toxic metabolic products (such as alcohol or organic acids) or extremes in environment (such as unfavorable pH or temperature) may become limiting under certain circumstances. Under test conditions which eliminate these factors what ultimately controls the population attainable in a culture?

Various studies have distinctly challenged the theory that reproduction may be controlled by specific autoinhibitory substances produced by the cells (e.g., Cleary et al., '35; Lockhart and Powelson, '53a). It seems certain that the only proof of this theory would be the isolation and characterization of inhibitory substances. Broom ('29) and Her-

shey and Bronfenbrenner ('37) have suggested that reproduction ceases as a result of substrate exhaustion, but this is not strictly true; it has been shown that after reproduction has stopped the limiting substrate is still available and the cells can continue to utilize substrates. The actual situation seems to be that suggested by Cleary et al. ('35): cell division is limited by the amount of nutrient *available per cell* under the test conditions.

The mechanism by which nutrient concentrations can control reproduction probably is complexed with the nature of equilibria of enzymatic reactions in the cells. It is conceivable that as fewer substrate molecules are adsorbed on and permeate the cell membrane the supply of essential intermediates in the metabolic pool of the cell becomes low and new equilibrium states are established in the chain of reactions by which substrates are converted to energy or products necessary for cell division. At present there is insufficient information to permit generalizations from this theory. Studies have provided a few clues as to the nature of the controlling mechanism in the coliform bacteria. Since reproduction in the culture ceases abruptly, not gradually (with a single substrate limiting growth), it seems likely that a specific reaction is involved. Also significant is the fact that abrupt cessation of cell division occurs whether the limiting substrate is the N source or the C and energy source.

It is also obvious that reactions permitting growth are not as sensitive to concentration of nutrients as reactions essential to cell division. These reactions apparently continue until new equilibria are reached as the substrate is more nearly exhausted. This period of growth is associated with increase in cell size, changes in composition of the cell and membrane permeability. Several recent reports in the literature indicate that changes similar to those we have noted here may be of general occurrence in cells subjected to a similar environmental situation. Sherrat and Thomas ('53) show a graph in which there is an increase in nucleic acid content of *Streptococcus faecalis* cells at the onset of

the stationary phase of growth, and Jeener ('52), working with a protozoan, has shown a rise in RNA content at this time. White ('53), studying *S. faecalis,* has found that heat resistance reaches a peak at the end of logarithmic growth and rapidly decreases in the early part of the stationary phase. Pinsky and Stokes ('52) have presented evidence for an increased ability of bacteria to form certain adaptive enzymes in the stationary phase. The demonstration of these changes at the stationary phase as well as in the early phases of growth adds strength to the view that such cellular changes may occur simply as a response to environmental conditions and not as a transformation of aged cells to "embryonic forms."

Regardless of whether or not subsequent events are analogous, various equilibria of the same enzymatic reactions may be involved in the control of cell division in the bacterial cell as in any other cell. As to the nature of these reactions it seems significant that the staling ability of a bacterial strain (that is, the relative amount of a limiting substrate required for reproduction) is correlated with its reducing capacity (Lockhart and Powelson, '53a). A high reducing capacity and the presence of reducing groups have been shown again and again to be necessary for the division of many types of cells (Needham, '42), and oxidation-reduction potentials are known to have a profound effect on growth and differentiation (cf. Moment, '52). The precise nature of the metabolic mechanisms which function here remain to be elucidated, but it appears likely that further study of these relationships in bacteria may help us to understand the factors controlling the growth of all cells.

SUMMARY

Cells of *Escherichia coli* in synthetic broth cultures at the onset of the stationary growth phase were observed to behave like the cells in the late lag and early logarithmic cultural phases, exhibiting a similar spectrum of cytological and physiological changes. Analyses of aerated cultures showed

that the limiting nutrient was not used up when reproduction ceased and that the cells continued to utilize the C and N substrates for a period thereafter. During the early part of this period as a result of growth without cell division the cells showed an increase in size, in content of nucleic acids, in RNA/DNA ratio, in phenol sensitivity, and in respiratory activity. The concentration of glucose restricting the growth of an organism appeared to be correlated with the staling activity and reducing capacity of that organism, according to a limited survey of strains of *E. coli* and *Aerobacter aerogenes.*

These data tend to support the theory that reproduction ceases in a bacterial culture when a specific equilibrium state is reached between the available substrate and certain metabolites in the reaction series leading to cell division. Reactions permitting synthesis of protoplasm appear to be limited by lower concentrations of certain nutrients. It is suggested that bacterial cultures that can be grown in very simple media and controlled by manipulation of a single nutrient may be employed profitably as test systems for detecting the specific reactions regulating reproduction and growth.

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ULTRAVIOLET EXCITATION OF A STRETCH RECEPTOR [1]

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THREE FIGURES

The crayfish possesses two stretch receptors on each side of each segment of the abdomen and several pairs in the thorax. Of each pair, one has a low threshold and a slow accommodation (RM_1) and the other a high threshold and a rapid accommodation (RM_2) (Wiersma, Furshpan and Florey, '52). The receptor with the low threshold and slow accommodation provides a readily available and excellent object for physiological analysis of the receptor process. Normally, it fires only when tension is exerted upon it, although a spontaneous discharge is found in some good preparations when the muscle fiber is relaxed or even limp. Since the receptor RM_1 adapts slowly, a continuous discharge at a relatively fixed frequency is obtained when a given tension is exerted upon it. The action of an extraneous agency other than tension upon the receptor therefore may be effectively studied. Ultraviolet light (UV) produces profound effects upon cells, yet it can be easily applied without otherwise disturbing the environment, therefore its actions upon the receptor was studied. The object of the experiments was the possibility of learning more about the process by which excitation of the discharge from the receptor cell is achieved as well as the chance to gather further information about the nature of UV effects upon cells.

[1] The authors are indebted to Dr. C. A. G. Wiersma for his active interest, encouragement of the study and criticism of the manuscript and to Dr. L. R. Blinks, Director of the Hopkins Marine Station, for facilities provided at the laboratory.

MATERIALS AND METHODS

The crayfish, *Astacus trowbridgei, Stimpson* collected in the Carmel River was used as the experimental animal. The RM_1 receptors from the second and third abdominal segments were used and the preparation was handled in the same manner as described in the above-mentioned paper. The muscle receptor organ was isolated and the muscle on which the dendrites of the ganglion cell ramified was lifted by forceps mounted on a rack and pinion device and the nerve was held on a micromanipulated electrode. The second electrode from the oscilloscope was immersed in van Harreveld crayfish solution containing the preparation. Records were obtained with a DuMont double-beam oscilloscope, model 322. The preparation was irradiated with U-shaped Westinghouse sterilamp which was suspended at from 5 to 12 cm from the preparation depending upon the intensity desired. Most of the experiments were carried out at a distance of 12 cm. The intensity of the light was measured with a Hanovia ultraviolet meter and at 12 cm was between 10–12 ergs /mm^2/second, the intensity rising to the higher value after about 5 minutes.

For photoreactivation either a GE H4 spot lamp was used through a no. 3060 Corning filter which cut off any short UV, or a projection lamp which was normally used in dissection, or both. Regardless of which light source was used, the beam of light was first passed through a water cell to remove the heat. The light from the projection lamp was found to have a slight inhibitory action on discharges from the sensory cell, that of the spot lamp did not.

EXPERIMENTAL

The irradiation of the stretch receptor with UV invariably produced an increase in the discharge frequency over that of the accommodation level, regardless of conditions under which it was performed. In all cases a brief induction period preceded the increase in frequency although sometimes an

almost immediate although slight increase in frequency was observed. Under the same conditions the control maintained an almost constant frequency of discharge over two hours of testing. That the effect of UV is directly upon the cell and not upon the medium was demonstrated by irradiating

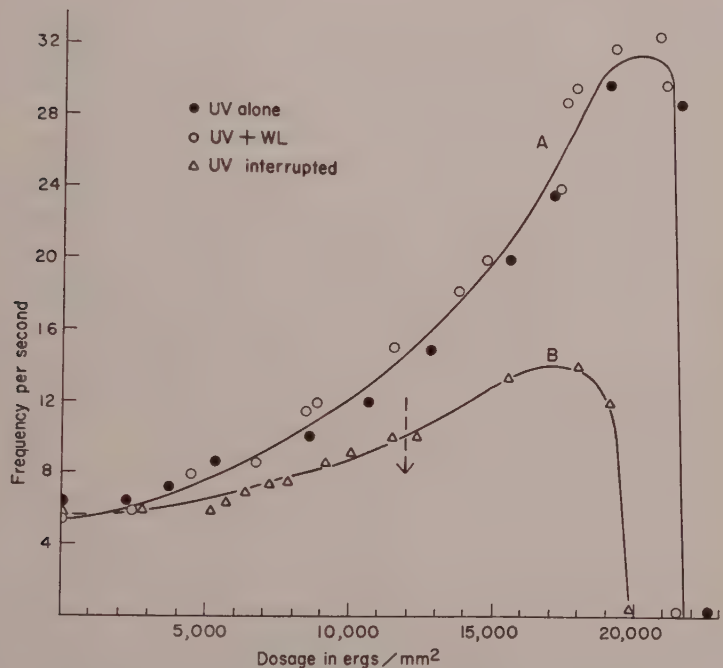

Fig. 1 Effect of ultraviolet light (UV) of intensity 12 ergs/mm²/sec. on the frequency of discharge of the muscle stretch receptor of the crayfish. (A) uninterrupted exposure; (B) interrupted at the arrow for 103 minutes between the first and second rounds of irradiation. The figure also shows the failure of white light (WL) applied concomitantly with UV to reverse the injurious effect of the latter.

the crayfish perfusion solution and then immersing the receptor in it. No change in frequency of discharge was observed in such an experiment.

If the preparation is continuously irradiated, the frequency of discharge increases continuously until a maximum is reached, after which it suddenly drops to zero (fig. 1). This

was true regardless of whether low or high intensity UV was used. A preparation irradiated until it has ceased firing will still usually respond to stretch, but the stimulus must be strong and rapidly applied, and the discharge consists of only a short burst of several impulses. After smaller dosages, the receptor is still capable of giving graded responses to stretch as seen in figure 2.

High intensity UV (fig. 3 C, D) induces a more rapid onset of high frequency discharge from the receptor, but the dosages required for evoking maximal frequency were much higher than when low intensity UV (fig. 3 A, B) was used. This suggests that either the absorption of light or some subsequent secondary thermochemical reaction is rate limiting. Temperature experiments were not performed, so that a choice cannot be made between the alternatives. The best results were obtained when low light intensities were used, consequently most of the experiments were performed with a light intensity of approximately 10 to 12 ergs/mm^2/sec.

In the course of the earlier experiments both the cell body and the axon leading from it were exposed to UV. Since the nerve action potential fell rapidly after a given dosage of UV had been applied, it appeared likely that the nerve fiber was injured.[2] Such injuries have been described by previous investigators (Audiat, '31; Hutton-Rudolph, '43; von Muralt, '53; Gasteiger, '53). This makes measurements of the changes in output frequency of the sensory cell difficult or impossible to ascertain. In most experiments only the receptor cell body and the parts peripheral to it (dendrites and muscle) were exposed, most of the axon being shielded either with a piece of black film or a glass coverslip. The latter, which is opaque to short UV, was used in most of the experiments described. As seen in figure 3 (A and C), the UV effect is much more

[2] In one experiment the cell body was covered with a coverslip and the axon was irradiated. The action potential rapidly fell to a low value and then vanished with little change in the frequency of discharge. This corroborates evidence of axonic susceptibility to UV from experiments in which the entire receptor cell preparation was irradiated. In subsequent experiments the axon was shielded by a coverslip.

striking when the entire preparation is exposed than when the axon is covered. This suggests an additive effect of the radiation on the different parts of the receptor, perhaps because of the greater area exposed to UV. Since the receptor

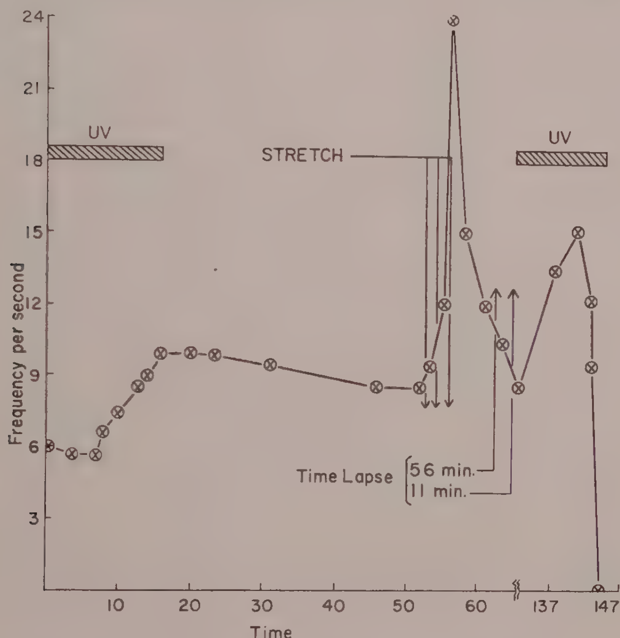

Fig. 2 Effect of stretch on the frequency of discharge from a UV-treated muscle stretch receptor of the crayfish. The UV dosage (intensity 12 ergs/mm²/sec.) can be obtained by multiplying the intensity by the time of exposure in seconds. The figure also shows the small degree of accommodation in the UV-treated preparation after cessation of exposure to UV. The downward pointing arrows indicate a stretch applied to the preparation with a micromanipulator to determine if the irradiated preparation would still respond. The upward pointing arrows show a time lapse of the duration designated, the time not being included on the abscissa. UV was applied only during the time lapse indicated by the cross hatched bars. Time on the abscissa is in minutes.

process was of main concern here, the subsequent experiments were performed with the axon covered by a glass coverslip to prevent direct passage of the UV. Experiments showed that this does not eliminate scattered radiation but greatly reduces UV injury to the axon.

In the next series of experiments the effect of a dosage of UV less than enough to elicit maximum frequency of discharge was studied. The results are shown in figure 1 B and 2. It is quite apparent that the discharge frequency of the receptor remains high for some time after the cessation of irradiation, but it does not increase. However, subsequent irradiation increases the rate of discharge up to a

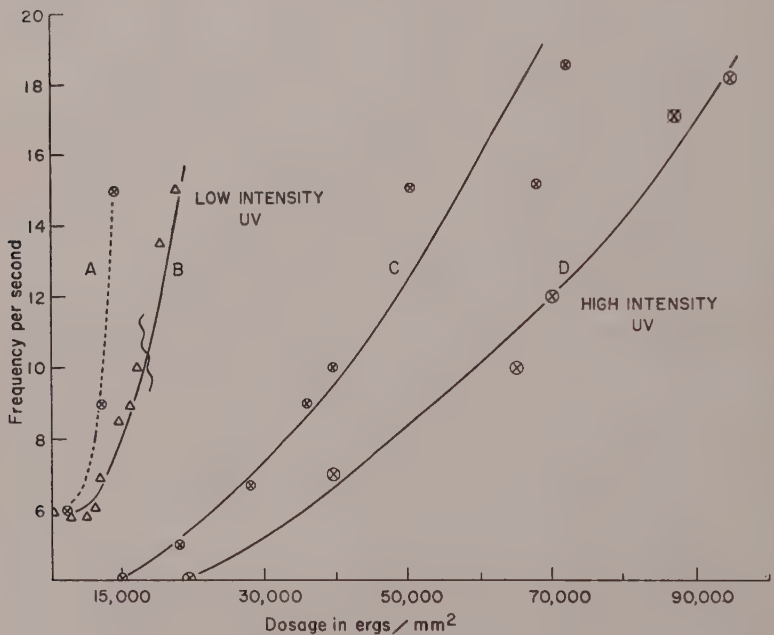

Fig. 3 Difference in action between high intensity (60–65 ergs/mm²/sec.). UV (A, B) and low intensity (10–12 ergs/mm²/sec.) UV (C, D) on the frequency discharge from the muscle stretch receptor of the crayfish. The figure also shows the greater action of the UV when the entire preparation was irradiated (A, C) than when the axon was shielded with a piece of cover glass (B, D). The wavy line in B indicates a brief pause in irradiation.

maximum after which it falls to zero in the characteristic way. The experiment is interesting because it shows that the UV has produced some change in state of the receptor cell which is relatively stable, such that the frequency of discharge continues at a relatively constant rate much like

a receptor preparation which has accommodated to a given stretch (fig. 2).

Attempts were next made to see if the preparation treated with UV could be photoreactivated [3] by illumination with visible light. A preparation which has reached a maximum frequency of discharge cannot be used for this purpose since it often ceases to fire so suddenly. Therefore a preparation irradiated with about one-half of the maximal exciting dosage was illuminated with the two lamps previously described. In no case was a significant decrease in frequency of discharge observed. Therefore in the final experiments, the receptor preparations being irradiated with UV were simultaneously illuminated with both of the sources of white light. Representative experiments are graphed in figure 1 A. No significant difference is observed between the preparation treated with both UV and white light and the preparations treated with UV alone. Photoreactivation of the receptor cell does not occur under the conditions in which these experiments were performed. Since in many organisms which have been given similar dosages of UV other processes were photoreactivated by illumination with the GE H4 lamp, the UV injury to the stretch receptor does not appear to be subject to photoreactivation.

Thiamin was found to restore the potential of a UV-treated frog sciatic nerve so that preparation continued discharging after the control had died. However thiamin HCl (0.02 to 0.06%) had no apparent beneficial effect on the UV treated receptor, nor did it protect the receptor from UV unless a layer thick enough to absorb the radiations was used.

An increased discharge frequency from the stretch receptor may be induced by application of acetylcholine (Wiersma,

[3] Some evidence for photoreactivation of the conduction process in frog sciatic nerve has been presented (Pierce, Miller and Giese, '53). The nerve stripped of the epineurium showed, on stimulation, a decreased spike potential after UV. On subsequent illumination with white light the spike sometimes increased in size or remained constant, whereas that of controls in the dark invariably disappeared. In the present study the attention was focussed on the receptor process rather than on conduction.

Furshpan and Florey, '52). This increase in frequency is abolished by the application of atropine. A possible explanation of the stimulating effect of UV on the stretch receptor was the secretion of acetylcholine induced by UV. If this were true the UV-treated preparation should cease firing or fire at a lower rate after atropine treatment. However, the receptor preparation excited to high frequency of discharge following UV radiation was unaffected when it was immersed in 0.04% atropine. Nor did immersion in atropine prevent the subsequent rise in discharge frequency when the treated rinsed preparation was later again irradiated. It is conceivable that atropine does not penetrate the receptor to reach a possible internal source of acetylcholine.

DISCUSSION

The increased frequency of discharge of a stretch receptor following exposure to ultraviolet radiation (UV) was always obtained and appears to be a constant feature of UV action upon this cell. This is not necessarily an expected result in view of the decreased spike potential of frog nerve exposed to UV.[3] The results may have some bearing on the nature of the excitatory process. Normally the receptor is excited to a higher frequency of discharge by tension or stretch. Since the high frequency discharge induced by UV results from injury to the cell, the suggestion arises that in normal excitation, some reversible injury of the cell excites the discharge.

There is little evidence of accommodation of the muscle receptor to UV excitation. Accommodation is also slow following slight mechanical stimulation. The interesting contrast in action of the two stimuli lies in the fact that the UV discharge continues after the light source has been turned off, whereas when mechanical stimulation ceases, the discharge frequency falls.

A delay in the rise of frequency was always found when a preparation was first irradiated. Since the entire receptor organ is encapsulated and the endings of dendrites lie in

the intercalated connective tissue, the UV may first affect either the tissue surrounding the receptor cell or the most readily reached parts of the receptor cell other than the region initiating the discharges. However the lag may be the time required for completion of the thermochemical reactions following the absorption of UV.

The relative susceptibility to UV of the three portions of the cell, the dendrites, the cell body containing the nucleus of the cell and the axon would be of considerable interest. Experiments were performed purporting to give such information for the latter two portions. However, the interpretation of the results is not unequivocal. When the axon of a receptor alone is exposed to UV, it soon ceases to fire, the height of the action potential falling rapidly the while. In this case the process being affected by UV is the conduction of the impulses and not necessarily the receptor process. When the axon is protected by a glass shield and the cell body alone is exposed to UV, an increase in frequency of discharge is obtained. However, it is impossible to ascertain to what degree the dendrites are being affected because it is not known to what extent they are reached by the UV. The fact that the cell body appears to tolerate irradiation better than the axon alone suggests a greater resistance of the cell body than of the axon. However, the effect of UV upon the appearance of spikes from the cell body is being compared with its effect on the conduction of the spikes along the axon. These phenomena are probably the result of different processes and are therefore not strictly comparable.

The failure to obtain photoreactivation of the receptor process is of considerable theoretical interest if the results can be taken at face value. It could be inferred that the materials responsible for discharge in the receptor cell are already available in quantities sufficient for very many discharges. Within the short term of the experiments performed it is unlikely that the system depends for its discharge upon the synthesis of additional materials. It is these synthetic processes which are especially subject to photoreactivation

(Kelner, '49, '53; Giese, '50; Swenson and Giese, '50). The change in state which results in a more rapid firing of the stretch receptor is perhaps sufficiently removed from contact with processes in which visible light reverses the action of UV and is therefore not susceptible to photoreactivation.[3]

SUMMARY

1. The frequency of discharge of the muscle stretch receptor (RM_1) of the crayfish *Astacus trowbridgei, Stimpson,* remains constant for long periods of time under constant tension. It is therefore a favorable object for studies of effects of agents such as UV upon the receptor process.
2. Following exposure to a given dosage of ultraviolet light (UV), after an induction period, the frequency of discharge rises steadily until a value of two to 5 times the original is obtained. Further irradiation is followed by a rapid fall to zero frequency of discharge.
3. A low intensity of UV is more effective per unit dose than a high intensity. Irradiation of axon and cell body is more effective than irradiation of cell body alone.
4. The irradiated receptor is capable of responding in essentially the normal way to stretch until the final fall to zero frequency. Following this it is practically insensitive to stretch.
5. Irradiation of crayfish perfusion solution alone is ineffective in exciting the stretch receptor to a higher frequency of discharge.
6. Attempts at photoreactivation by visible light were unsuccessful regardless of the time at which the light was applied.
7. Thiamine had no effect on the frequency of discharge of a normal receptor. It neither induced recovery nor protected the cell from subsequent irradiation with UV.
8. Acetylcholine is known to cause an increased frequency of discharge which is counteracted by atropine. However, atropine had no comparable effect upon a preparation excited to high frequency of discharge by UV.

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EFFECTS OF THE INJECTION OF SNAKE VENOM CONTAINING LECITHINASE A INTO PHYSARUM POLYCEPHALUM

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The submicroscopic structures of protoplasm may be assumed to exist in part as aggregates, some of which are visible with the microscope. Entering into the formation of these aggregates is a great variety of substances, i.e., proteins, lipids, glucosides, and inorganic ions. Basic to the nature of protoplasm appears to be its ability to undergo reversible sol-gel transformations. These may be observed by noting variations in protoplasmic flow in the slime mold *Physarum polycephalum*.

Haas ('53) injected a variety of enzymes into this slime mold and has observed directly changes in protoplasmic streaming. For example, he found that certain proteinases, collagenase, some polysaccharases, and ribonuclease produce characteristic changes in protoplasmic activity, which reflect the action of these enzymes on the submicroscopic structure of protoplasm. He interpreted solation and gelation as a response to enzymatic activity which causes a loosening and reestablishment of specific bondings. Heilbrunn ('52) especially has emphasized the role of calcium in causing gelation and solation of protoplasm. However, it is not clear how changes in the amount of free or protein-bound calcium may be controlled *in vivo*. The work reported here is an attempt to relate some possible enzymatic activities to changes in the local concentration and state of calcium.

MATERIALS AND METHODS

Enzymes and salt solutions were injected into the slime mold *Physarum polycephalum* through a micropipette. The

mold was then observed under the microscope for one to three hours. Comparisons were made between the preparation injected as above and the pre-injection activity of the mold and the activity of a control removed from the mother plasmodium at the same time as the experimental. In addition to these uninjected control molds, others were injected with double distilled water or with salt solutions. It was found to be important that all solutions be made up with double distilled water.

The slime mold was grown on moist filter paper with autoclaved oatmeal as the nutrient medium. Between one and one and one-half hours before the injection small portions of the original colony, which was grown on filter paper, were cut off and placed in a drop of water on a cover slip. When they began to spread they were inverted over a moist chamber and injected. The molds were kept moist during the entire observation period.

The first substance injected was a solution of Russell's Viper venom which contains lecithinase A and other enzymes. In subsequent experiments, these other enzymes were excluded. As the action of lecithinase A is to release a fatty acid from lecithin and cephalin (Wittcoff, '51), one would assume that this fatty acid would form an insoluble calcium soap. In order to test this hypothesis the next substance to be injected was sodium stearate. Since the effects of lecithinase A and sodium stearate could be attributed to the withdrawal of protoplasmic calcium it was thought desirable to inject citrate which combines with calcium to form an unionized complex. Calcium chloride was injected to determine the effects of excess calcium upon the protoplasm.

Russell's Viper venom was purchased from Ross Allen's snake farm. The snake venom was injected in concentrations ranging from 10^{-3} to 10^{-6}. This injection contained lecithinase A and all other enzymes present in Russell's Viper venom.

All other enzymes besides lecithinase A were destroyed by heating the venom solution at 100°C. for 15 minutes (Braganca and Quastel, '52).

All enzymes including lecithinase A were destroyed by autoclaving the venom for 30 minutes at 15 pounds pressure (Roy, '38).

The lecithinase A activity in each of the above preparations was tested by the hemolytic method of Slotta and Szyzka ('38).

Concentrations of the solutions were as follows: sodium stearate 0.003 M, calcium chloride 0.02 M, sodium chloride 0.003 M, and sodium citrate 0.01 M.

RESULTS

(a) Russell's Viper venom. Following the injection of venom containing lecithinase A and all other enzymes of Russell's Viper venom the solated material in the organisms was increased, as judged by the greater amount of fluid protoplasm in the pseudopods particularly. The reaction observed varied in intensity only with the concentration of the venom.

Upon injection the protoplasm became paler, and streaming flow stopped for about 20 minutes. Flow in other areas somewhat removed from the site of injection was sometimes inhibited for a short time if the solutions were injected directly into an active channel. The channel clotted, but flow was soon reestablished around this region; sometimes new channels were formed. At the end of the period when flow was reduced or had stopped, flow within the entire plasmodium was resumed and rapidly became faster than normal. The increased rate of flow was not observed in all areas at once. When the streaming flow increased, the normal type of pseudopodial flow which is confined to discrete channels was replaced by a pulsating flow which filled the entire pseudopod. Channels in areas of the plasmodium which were not immediately affected by the injected material widened, and the rate of protoplasmic flow in them increased. Hyaline protrusions became more numerous over many areas of the plasmodium, especially in the region of the injection; some of these protrusions developed into pseudopodia. The af-

fected pseudopodia increased in size after injection and many spread widely. Many of the bud areas close to the injection site increased in size and later regressed.

(b) Russell's Viper venom containing active lecithinase A. Other enzymes present in the venom were inactivated as described above. The amount of solated material increased markedly after the injection of this active preparation into the plasmodium. The reaction was identical with that caused by untreated venom, possibly even more intense.

(c) Russell's Viper venom having no enzymatic activity (see page 205). Following the injection of this inactive preparation, flow in the plasmodia was not notably altered as compared with controls.

(d) Sodium chloride. Sodium chloride solution caused early clot formation. Temporarily the hyaline layer became thicker after injection (but this was slight as compared with that seen after the injection of sodium citrate). The preparation appeared normal after a few minutes.

(e) Sodium stearate. Sodium stearate solution caused an increased amount of flowing protoplasm with a later solation of almost the entire plasmodium.

Upon injection, or shortly thereafter, the site of injection became opaque and no flow was visible. Budding areas were not affected. In the distal parts of some buds the protoplasm appeared to flow unchanneled into hyaline protrusions. After about 40 minutes many areas of the plasmodium appeared paler. Soon, flow resembling that seen after the injection of lecithinase A occurred in some large channels. In about 100 minutes most of the mold lacked pigment and the relatively few granules remaining were discernable as discrete particles in a liquid-filled sac. In some areas channels were visible but their contents were static.

(f) Sodium citrate. The injection of sodium citrate increased the amount of flowing protoplasm in the plasmodium.

Sodium citrate caused excessive budding at the site of the injection, with extensive spreading of the plasmodium. Later the site of injection darkened and flow ceased briefly in many

areas of the plasmodium. Rhythmic flow soon reappeared accompanied by some spreading of the plasmodium. Within 20 minutes after the injection flow resembled that seen after injection of venom or lecithinase A. Forty-five minutes after injection the region of the injection was marked by an increased amount of flowing material and a rapid spread of pseudopodia. The moving protoplasm was confined to numerous small channels separated by thin walls. Concomitantly with the local change, the plasmodium as a whole spread, though not to the same extent as the injected area. After about 85 minutes some regression sites were visible, while secondary budding around the injection site continued.

(g) Calcium chloride. The injection of calcium chloride reduced the activity of the plasmodium.

After injection flow near the injection site stopped. Three to 5 minutes later the protoplasm in the affected site darkened. Protoplasmic flow in the other regions of the plasmodium remained normal for about 20 minutes. After this time some of the bud areas regressed. About 35 minutes after the injection movement in the primary buds stopped and secondary buds began to appear along the injected limb. About 15 minutes later most of these had been resorbed. The remaining buds showed little protoplasmic movement. Another spurt of activity occurred 70 minutes after the injection, with renewed budding along the injected limb; these buds remained small and were soon resorbed. During the entire period of observation the protoplasm in the main channels continued to flow slowly.

(h) Double distilled water. Flow was relatively normal after the injection of double distilled water except for a short post-injection period of clotting when the protoplasm appeared paler. With the resorption of the clot the plasmodium resembled uninjected controls.

DISCUSSION

The injection of substances having lecithinase A activity, of sodium stearate, and of sodium citrate all appeared to

have similar effects upon the protoplasm of the slime mold *Physarum polycephalum.* All of these substances caused a decrease in protoplasmic viscosity which may be interpreted as a gel to sol transformation. They also caused, to different degrees, an increase in spread of the plasmodium and in rate of flow of the solated protoplasm. The common effect of lecithinase A, fatty acid, and citrate may be understood if one postulates that in each case calcium is removed progressively from its combination with proteins and an insoluble or unionized compound formed. Lecithinase A causes the release of a fatty acid from lecithin and cephalin. This fatty acid may form an insoluble soap with the calcium of the protoplasm, thus binding it and removing it from its structural relationships. The stearate salt is apparently capable of direct formation of a calcium soap in the protoplasm with the same consequences. The citrate ion combines with calcium to form an unionized soluble complex and thus shifts the equilibrium in the direction of removing calcium from its protoplasmic combinations.

The injection of a calcium salt into the mold reverses the effects of the calcium-binding substances and there is an apparent sol to gel transformation with a consequent increase in protoplasmic viscosity. The rate of flow of the protoplasm decreases and all flow is in discrete channels; furthermore spread of the plasmodium is at a minimum.

From the preceding analysis it might be concluded that calcium is integrally related to the sol-gel transformations seen in protoplasm at least under certain experimental situations. The decreased viscosity (solation) observed with the binding of calcium may be accounted for by the fracture of the bonds between adjacent protein aggregates. This fracture would permit the contraction of the protein chains which Frey-Wyssling ('53) believes to be involved in protoplasmic flow. The fracture of many of these bonds concurrently would cause the massive flow actually observed in the mold. On the other hand, an increase in ionic calcium concentration within the mold would decrease the flow, likewise as observed in this study.

The hypothesis that calcium influences gelation of protoplasm has in the past stumbled over an important difficulty, i.e., the mechanism of effecting a transformation of gel to sol under controlled conditions. The work reported above furnishes a basis for a hypothesis that might resolve this difficulty. The hypothesis is that calcium may be shifted from one portion of protoplasm to another as a result of enzymic activity. The latter consists in the release of excess fatty acid by the action of lecithinase A on lecithin or cephalin. The excess fatty acid combines with calcium derived in part from its combination with gelled protoplasm. The calcium-free protein coincidentally becomes more fluid (sol). The hypothesis may be made even more general in that (1) other enzymes may contribute to the formation of excess free fatty acid, (2) still other enzymes may contribute to citrate formation. (3) Other ions, e.g. magnesium, may be similarly dissociated from protein combinations.

SUMMARY

1. The injection of lecithinase A into the slime mold *Physarum polycephalum* augmented the activity of the plasmodium. The gel matrix was decreased. The rate of protoplasmic flow and forward flow of the plasmodium was accelerated. Sodium stearate and sodium citrate caused similar changes in the activity of the mold.

2. Calcium chloride caused a decrease in the activity of the mold marked by decreased budding, reduced rate of protoplasmic flow, and rapid regression of newly formed buds.

3. It is proposed that the effects on sol to gel transformations caused by the injection of lecithinase A, fatty acid, and citrate may be due to the binding of calcium and its withdrawal from combination with proteins.

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TEMPERATURE-PRESSURE EXPERIMENTS ON AMOEBA PROTEUS; PLASMAGEL STRUCTURE IN RELATION TO FORM AND MOVEMENT [1]

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FIVE FIGURES

INTRODUCTION

Because high hydrostatic pressure consistently exerts a very distinct solating action upon protoplasmic gel structures, this agency has provided an excellent method for analyzing the physiological role of sol ⇄ gel reactions in several kinds of cells (Marsland, '39, '42 and '50; and Pease, '40 a and b). In the amoeba, for example, it was found that the inhibition of movement and the collapse of the pseudopodia, which are observed at high pressures, result from a critical weakening of the gel structure of the plasmagel system (Marsland and Brown, '36 and Brown and Marsland, '36). Similarly in dividing eggs, it was shown that the inhibition of furrowing by high pressure is related to a weakening of the plasmagel structure of the egg cortex — as measured by the pressure-centrifuge technique (Marsland, '39 and '50).

Protoplasmic systems, apparently, conform to the criteria of Freundlich ('37) for gels of the methyl cellulose type (type II). In such systems, the gelation process represents an endothermic reaction which exhibits a significant volume increment ($+\Delta V$). From the Le Chatelier principle, therefore, it would be expected that the equilibrium of such a system

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would be shifted in the sol direction not only by *increasing pressure* (Marsland and Brown, '42), but also by *decreasing temperature.* This expectation has been justified by some recent work on dividing eggs (Marsland, '50 and Marsland and Landau, '54). These experiments showed that the gel structure of the cortical plasmagel layer of the egg cell is distinctly weaker at lower temperatures (as well as at higher pressures) and that the weakening of the gel structure is parallelled by a weakening of the furrowing potency of the cell.

Clear indication that the plasmagel of the amoeba represents a type II system is provided by the previously cited pressure experiments of Brown and Marsland ('36). However, contradictory evidence can be found in the work of Thornton ('32), who reported that an *increase* in the plasmagel "viscosity" occurs at lower temperatures. One aim of the present experiments was, therefore, to resolve the foregoing question by making a systematic study of the temperature and pressure characteristics of the plasmagel system in relation to the form and movement of the amoeba. Such a study is needed, moreover, to provide a basis for further studies on the metabolism of protoplasmic gelations.

MATERIALS AND METHODS

Culture technique. The method of Brandwein ('35), modified by the elimination of the agar, gave an excellent supply of vigorous cultures, which were buffered at pH 6.9 and maintained at 18°–20°C. No significant variation could be noted as to the behavior of specimens derived from different cultures. Accordingly, the cultures were used rotationally to avoid the depletion of any one.

Other methods. The temperature control housing and the microscope-pressure chamber, which have been described by Marsland ('50), permitted the amoebae to be observed at magnifications up to 600 diameters while subjected to pressures up to 16,000 lbs./in.^{2}, at temperatures ranging between 5° and 30°C.

A pressure-centrifuge head, similar to the one described by Brown ('34) was used for the measurements of the plasmagel strength. A needle valve permits a sealing-in of the pressure on one side of this head while the other side contains the control specimens at atmospheric pressure. The centrifuge was kept inside the temperature housing, — which made it possible to perform the centrifugations at any temperature and pressure within the specified ranges.

In all the experiments the centrifugal force was standardized at 5700xG. A constant voltage transformer was employed in the power line, so that fluctuations in the centrifugal speed (which was checked periodically with a stroboscope) were negligible. Also a 2 mm layer of 8% sucrose solution was placed at the bottom of each centrifugal chamber, in order to protect the specimens from mechanical deformation.

Experience soon showed that an examination of the living amoebae after centrifugation failed to allow for a critical appraisal of the centrifugal displacement, because the specimens quickly resumed amoeboid activity, which quickly redistributed the cytoplasmic granules. Therefore, the specimens were subjected to heat fixation, accomplished by dropping the centrifuge head into boiling water for two minutes, immediately following the centrifugation. Then the amoebae from both the pressure and the control chambers were pipetted onto microscope slides and examined at a magnification of 440 diameters. Excellent fixations were thus obtained and ample time was allowed for carefully judging the stratification.

Temperature equilibration. The following procedure assured a complete equilibration of the specimens at each experimental temperature. About 150 amoebae were pipetted from the culture dish into a 15 cm^3 centrifuge tube and gently spun down in a hand centrifuge. Then 0.1 cm^3 of the resulting dense suspension at the bottom of the tube was transferred into 10 cm^3 of Brandwein solution at the proper temperature in the temperature control chamber. These amoebae were kept at the experimental temperature for one-half hour and then

transferred to the centrifuge chambers or to the microscope pressure chamber, which previously had been equilibrated to the proper temperature in the control housing. During the experiments variations from the selected temperature did not exceed $\pm$ 0.5°C.

EXPERIMENTAL RESULTS

Pressure effects on form and movement. Although both temperature and pressure were found to have a very decided influence on the form and movement of the amoeba, first to be considered will be the pattern of changes imposed by pressure alone. This pattern was described previously by Marsland and Brown ('36), who worked at room temperature (20°–22°C.). The present observations, which were carried out at the accurately controlled temperature of 25°C., essentially represent a confirmation of this earlier work.

At 25°C. the equilibrium form, observed when the specimens are kept at pressures between 1000 and 1500 lbs./in.^{2}, does not differ very radically from that of the control specimens. There are however, fewer pseudopodia and these tend to be more slender and perhaps a little longer. Few of the specimens display more than two pseudopodia and many are unipodal in form. The rapidity of movement of the specimens is not appreciably changed. In most of the pseudopodia, however, the plasmagel sheet (Mast, '26) appears to be absent, so that hyaline tips are seldom observable. Moreover, the direction of advance undergoes frequent though slight changes as a result of small ruptures occurring in the wall of the pseudopodia, close to the distal tips.

At pressures between 2000 and 3000 lbs./in.^{2} the pseudopodia are not only more slender, but also distinctly shorter than usual. Movement is definitely reduced and changes of form are usually slow. Many of the pseudopodia display bulbous tips, formed by the lateral rupturing of the distal parts of the pseudopodial walls.

At 3000–4000 lbs./in.^{2} the amoebae are unable to sustain any well extended pseudopodia. Those present when the

pressure is first built up, quickly develop bulbous tips and then slowly undergo retraction, so that after about 10 minutes the specimens show a compact though irregular form. Numerous new pseudopodia continue to form very slowly, but these are drastically reduced both as to length and diameter. In fact, such compact specimens appear to be covered with tiny bump-like pseudopodia, each containing only a very few granules. Some barely perceptible retraction and new formation of these miniature pseudopodia continues, but beyond this, the specimens are inert and devoid of orientation.

The response at 4000–6000 lbs./in.^{2} tended to vary according to the form displayed by the particular specimen at the time when the pressure was built up. If the form was relatively compact, i.e. without well extended pseudopodia, the specimens slowly (within 5–10 minutes) rounded up, — into perfectly motionless spheres at 5000–6000 lbs./in.^{2}, — or into slightly irregular spheroid masses, at 4000–5000 lbs./in.^{2}. But when well-extended pseudopodia were present, these collapsed abruptly — so that each cylinder quickly changed into a sphere, which sometimes remained attached to the basal remnant of the pseudopodium, but often pinched off as a completely separate body (see fig. 1). Usually only the distal half, approximately, of the pseudopodium was involved in the collapse, but at pressures approximating 6000 lbs./in.^{2}, even more of the pseudopodium participated, and the pinching off was observed more frequently. Following the rapid pseudopodial collapse, the specimens maintained at 5000–6000 lbs./in.^{2} slowly rounded up, into a single spherical mass, if pinching off had not occurred, or into two or three spheres, if one or two pseudopodia had been pinched away.

The complete rounding of a specimen gave a clear indication of the pressure level at which it could no longer maintain any pseudopodia. However, some variation was encountered as to the behavior of the individual specimens. Therefore, a total of at least 200 amoebae were used at each temperature-pressure level and the criterion employed in determining the minimum pressure required to prevent the formation

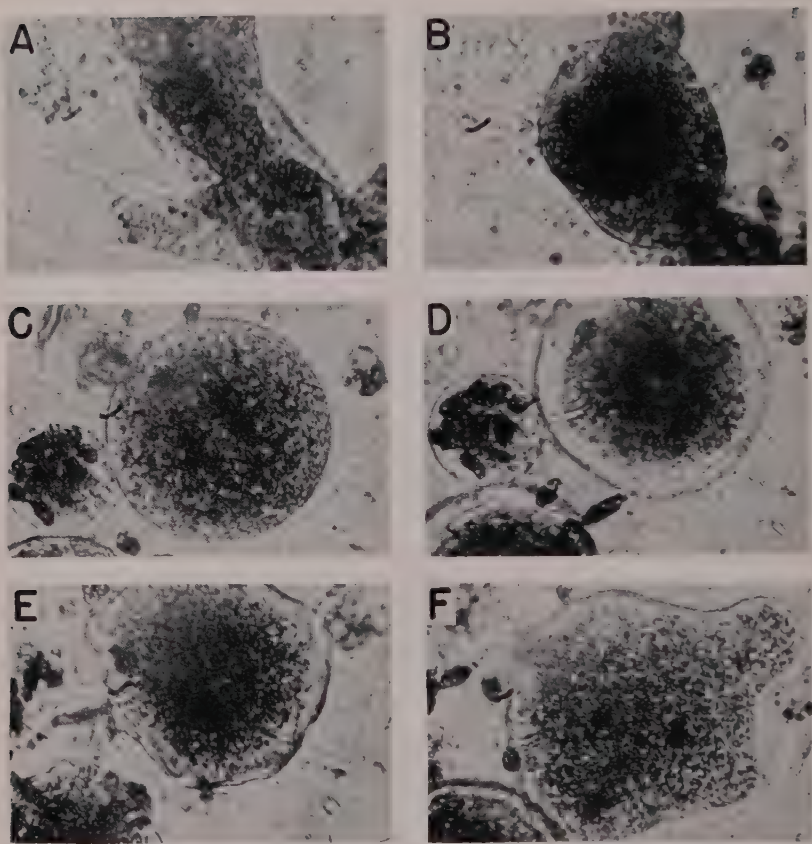


Fig. 1 Changes of form and activity in Amoeba proteus during and after exposure to a pressure of 6000 lbs./in.^{2} at 25°C.

A: Normal form at atmospheric pressure.

B: Same specimen at 6000 lbs./in.^{2}, maintained for 5 minutes. Note the rounded form of the main cytoplasmic mass and the pinched-off part of the large pseudopodium (above).

C: Fifteen minutes later, pressure still maintained. Specimen has rotated about 90 degrees. Note complete rounding of the main mass and of the pinched-off part of the cytoplasm, (which now lies to the left).

D: Fifteen *seconds* after pressure was reduced to the atmospheric level. Note the marked contraction of the granular cytoplasm (plasmagel) and the development of a broad hyaline zone between the granular cytoplasm and the cell membrane.

E: Ninety seconds after decompression. Note first signs of amoeboid activity.

F: Seven minutes after decompression. Amoeboid activity now quite vigorous.

and maintenance of the pseudopodia was a complete rounding up of approximately 75% of the specimens within 20 minutes (see table I).

The pressure level at which protoplasmic streaming stopped, on the other hand, could not be determined very accurately. Whenever the pressure was raised abruptly by 1000 lbs./in.² or more, there was a temporary cessation of flow, but streaming started again in 40–50 seconds, provided the pressure level was not too high. In the range around 4000 lbs./in.² some streaming must have been maintained, since the miniature pseudopodia continued to be formed and retracted. But the slow rate at which the granules moved made it difficult to perceive any movement. The best that can be said, therefore, is that some feeble streaming continued up to a point within about 1000 lbs./in.² of the pressure required to produce a complete rounding of the specimens.

TABLE 1

Effect of temperature on the critical pressure above which the pseudopodia of Amoeba proteus cannot be formed or maintained

TEMPERATURE	PRESSURE	NUMBER OF SPECIMENS OBSERVED	PERCENTAGE OF SPHERICAL SPECIMENS
	(lbs./in.²)		
	2000	209	5
10°C.	3000	225	76
	4000	218	100
	3000	200	6
15°C.	4000	224	75
	5000	216	90
	4000	213	36
20°C.	5000	221	77
	6000	217	90
	5000	218	61
25°C.	6000	230	89
	7000	208	100

Influence of temperature. Similar experiments, performed at 20°, 15° and 10°C. gave an interesting series of parallel

observations. Generally speaking, comparable changes of form occurred, except more slowly. But what seemed of even greater significance was that the pressure level required to induce each change became lower and lower as the temperature was reduced.

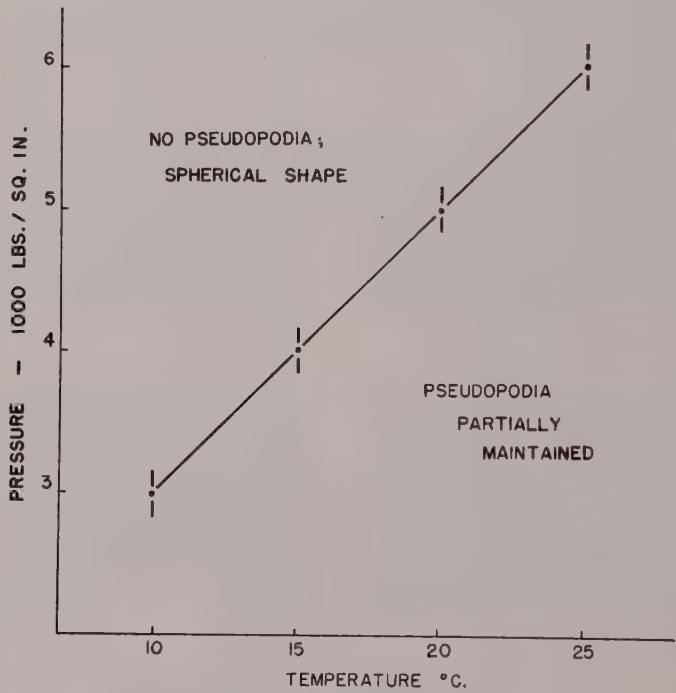


Fig. 2 Relations of pressure and temperature to the capacity of Amoeba proteus to form and maintain pseudopodia. Note that the critical pressure above which the cells cannot form or maintain any pseudopodia is increased by 1000 lbs./in.^{2} with each temperature increment of 5°C.

As is shown in table I and figure 2, the minimum pressure required to achieve our criterion for the rounding of the amoebae into quiescent spheres fell off progressively — from 6000 lbs./in.^{2} at 25°; to 5000 at 20°; to 4000 at 15°; and to 3000 at 10°C. At low temperature, the rounding process was more sluggish, requiring some 15–20 minutes for completion at 10°, as opposed to 5–10 minutes at 25°C. Moreover, the in-

termediate forms, in which reduced pseudopodia persisted, were similar to those described for 25°, except that the pressure level at which each equilibrium form was reached showed a reduction of approximately 1000 lbs./in.^{2} each time the temperature was reduced by 5°C.

Another difference between the high and low temperature observations is provided by the pinching-off phenomenon. This became less frequent and distinct at lower temperatures. The pinched-off part frequently involved $\frac{1}{4}$ to $\frac{1}{8}$ of the whole cytoplasm at 25° and 20°. Whereas at 15°, the pinched-off spheres were very small fragments; and at 10° pinching-off almost never occurred. Also, since the pseudopodia were less elongate at lower temperatures, the abrupt type of pseudopodial collapse did not occur below 15°C. Instead, the rounding of the specimens took place very slowly in barely perceptible stages, presumably because of an increased viscosity of the plasmasol, induced by the lower temperature.

Decompression effects. On sudden decompression, the rounded specimens, after they had been kept for some time at high pressure, displayed a very striking reaction (see fig. I, D). Within 10–15 seconds they developed a broad hyaline zone between the cell membrane and the granular plasmagel layer, which previously had remained in contact with the cell surface. Apparently this reaction represents a sudden generalized contraction of the plasmagel. This layer seems to detach itself from the membrane over the entire cell surface, and to contract, squeezing forth a clear fluid which fills the broadening space between the membrane and the outer boundary of the granular plasmagel (fig. I, D). Thus finally the still rounded specimen displayed three concentric zones: (1) the central contracted granular mass (which accounts for about 50% of the total volume), (2) the broad zone of clear fluid (which surrounds the granular gel), and (3) the cell membrane (which remains in its original position). Such a form was displayed, moreover, not only by the entire rounded specimens but also by the pinched-off spheres, unless these

were very small, or unless they contained numerous food masses (fig. I, D).

Following this sudden contraction, usually within 10–20 seconds, the amoebae commence to show signs of renewed activity. The deep-lying granular cytoplasm begins to break out into the hyaline zone in several places, bulging the cell membrane in an irregular and dis-oriented fashion. Soon, however, well developed pseudopodia begin to form, and 4 to 5 minutes later, normal locomotion reappears. Some semblance of normal pseudopodial activity is likewise displayed by some of the larger pinched-off fragments, but the small ones retain their inert spherical form.

The decompression contraction phenomenon is most plainly observed at higher temperatures — where the pressure required to cause a rounding of the specimens is likewise high. Also the reaction is more pronounced as the compression period is lengthened, reaching a maximum in about 20 minutes at 6000 lbs./in.^{2}. At lower temperatures, where the critical rounding pressures are lower, the decompression reaction is weaker. In fact, at 10°/3000 lbs./in.^{2}, no contraction could be observed. At this temperature, however, a small but decisive contraction did occur after a pressure of 7000 lbs./in.^{2} had been maintained for 20 minutes, — but such a combination of high pressure and low temperature undoubtedly resulted in some irreversible damage, since many of the specimens subsequently failed to resume normal locomotion.

A few experiments were done, in which Amoeba dubia was used in place of A. proteus; and the results of one of these are shown in figure 3. Generally speaking the two species behave similarly, except that A. dubia, with its smaller more numerous pseudopodia, displayed the "pinching-off reaction" less frequently, and the pinched-off fragments tended to be much smaller. Moreover, the decompression-contraction phenomenon was generally less striking in A. dubia, as may be seen in figure 3, D.

Temperature-pressure effects on plasmagel structure. The alterations of form imposed by increasing pressure and de-

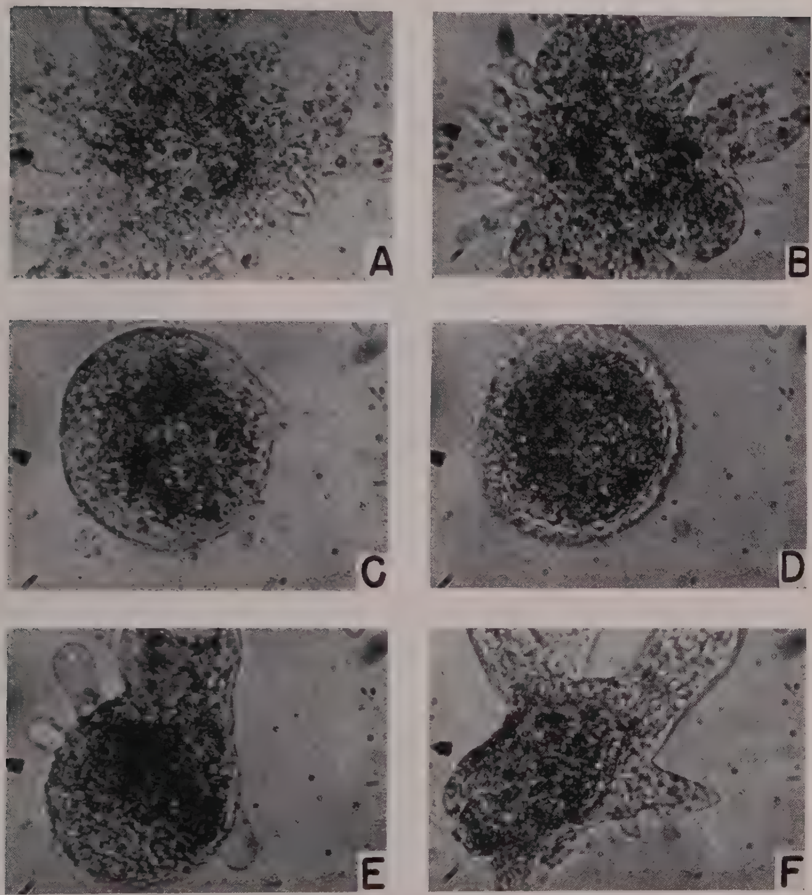


Fig. 3 Changes of form and activity in Amoeba dubia during and after exposure to a pressure of 6000 lbs./in.^{2} at 25°C.

A: Normal form at atmospheric pressure.

B: Ten seconds after pressure was raised to 6000 lbs./in.^{2}; pseudopodia beginning to retract.

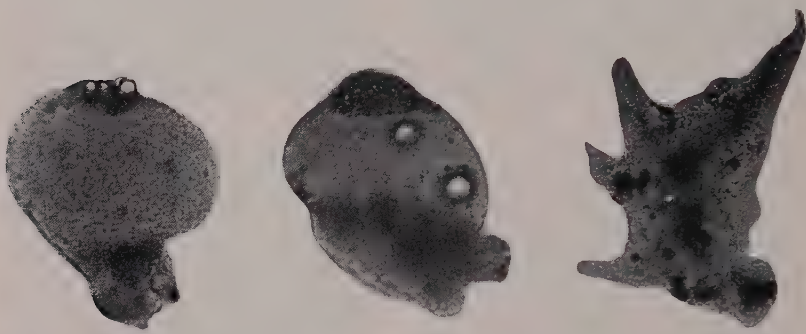
C: Pressure maintained for 5 minutes; rounding of specimen almost complete.

D: Ten seconds after decompression. Note contraction of granular cytoplasm away from the cell membrane.

E: Fifteen seconds later. Amoeboid activity recommencing.

F: Five and a half minutes later. Amoeboid activity now quite vigorous.

creasing temperature all seemed to indicate that the plasmagel structure was being weakened by the experimental treatment. However, direct quantitative evidence was lacking, and therefore a systematic series of centrifugal measurements of the



A B C

Fig. 4 Displacement of granular elements of the plasmagel of Amoeba proteus pressure-centrifuged at different temperatures. Specimens heat-fixed immediately following centrifugation.

A: Typical specimen, centrifuged for 15 seconds under a pressure of 6000 lbs./in.^{2} at 25°C. Oil cap, above; zone of heavy granules, below; and developing hyaline zone, between. Presence of numerous fine granules in "hyaline zone" indicates that this specimen has not quite reached the standard displacement endpoint. Note oil droplets which sometimes exude from the oil cap in heat-fixed specimens.

B: Typical specimen, centrifuged for 15 seconds at 6000 lbs./in.^{2} at 15°C. Absence of granules in hyaline zone indicates that this specimen has reached the standard endpoint. Oil droplets (out of focus) are not in the cell. Width of heavy granule zone varies considerably from specimen to specimen, depending on the granular content.

C: Control specimen, centrifuged simultaneously with specimen B, but at atmospheric pressure.

gel strength as a function of both temperature and pressure was undertaken.

The centrifugal method of measuring the relative consistency of the plasmagel layer is based on the usual assumption that Stokes' law provides a useful approximation when applied to protoplasmic gel systems. Quite obviously the resistance to displacement of visible granules through

a gel cannot be regarded as an index of *viscosity* in a strict physical sense. However, assuming that an adequate and uniform centrifugal force is used and that the density differential between the granules and the surrounding medium is not altered by the experimental treatment, it seems valid

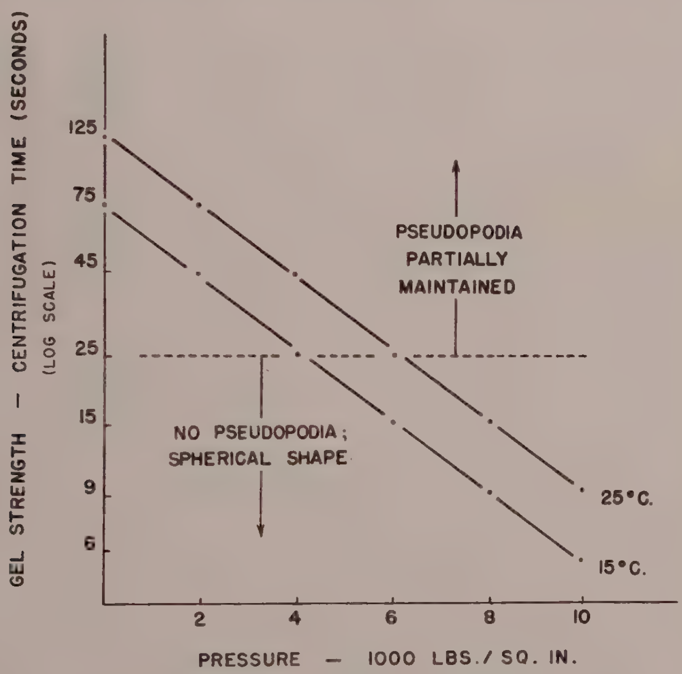


Fig. 5 Structural strength of plasmagel of Amoeba proteus plotted as a function of pressure at 15° and 25°C. Note that the two curves are parallel and that the critical gel strength, below which no pseudopodia can be maintained, is the same at both temperatures. Each point represents the average of values obtained in at least 6 determinations.

to take the centrifuge time required to produce a standard displacement of the granules through the plasmagel layer as an index of the "relative consistency," "stiffness" or "structural strength" of this part of the protoplasm.

In a fairly strong centrifugal field, such as the one (5700 × G) used in these experiments, the granular elements of the plasmasol part of the cytoplasm are displaced into

the extremities of the amoeba very quickly. The movement of granules embedded in the plasmagel, on the other hand, is much slower, so that no clear hyaline zone, intervening between the centripetally placed oil cap and the centrifugally located granular zone, appears until the granular elements of the plasmagel layer have undergone an adequate displacement. Consequently, the minimum time required to produce a clear hyaline zone, such as is shown in figure 4, B, may be taken as a relative index of the structural state of the plasmagel layer, provided that a uniform centrifugal force is utilized in the different experiments.

The effects of both temperature and pressure on the plasmagel structure of Amoeba proteus are presented graphically in figure 5. Here it may be seen that the gel strength displays an exponential decrease with rising pressure and, since the two curves are plainly parallel, the rates of decrease at 15° and 25°C. are the same. Moreover, it is possible to establish an equivalence between the oppositely directed effects of temperatures and pressure, — each increment of 2000 lbs./in.^{2} being just adequate to counterbalance the gelational increase produced by raising the temperature by 10°C., — throughout the range of pressures employed.

In relating the measured temperature-pressure effects on the gel structure to the changes in amoeboid form observed under parallel conditions, figure 5 shows that, regardless of the specific pressure-temperature value, the amoebae lost their capacity to maintain pseudopodia and displayed a completely rounded form, only when the structural strength of the plasmagel layer was reduced to a certain critical level. This level, expressed in terms of the atmospheric gel strength value obtained at 25°C., came out as 20%; or in terms of the scale used in figure 5, at the 25 unit position. Thus at 25°C., where the initial atmospheric gelational state was high, complete rounding did not occur until the pressure reached 6000 lbs./in.^{2}; whereas at 15°C., where the initial gel strength was lower, rounding was complete at 4000 lbs./in.^{2}.

In studying the centrifuged specimens, large differences in the plasmagel strength are easy to detect, since the control amoebae show very little displacement even of the coarser granules when the experimental specimens have reached the standard end-point. This may be seen by comparing the specimens presented in figure 4, B and C — which demonstrate the drastic weakening of the gel system imposed by applying a pressure of 6000 lbs./in.^{2} at 25°C. But an appraisal of small differences of gel strength requires that very careful attention be given to the finer granules which are not displaced from the hyaline zone until the end-point is being reached. This may be seen in the specimens of figure 4, A and B — which illustrate the smaller increase of gel strength incident to raising the temperature from 15° to 25°C. Here the presence of numerous fine granules in the developing hyaline zone of A indicate that this 25° specimen had not quite reached the end-point; whereas B, in which the hyaline zone is devoid of granules, was judged to be at the end-point.

DISCUSSION

In general, the alterations of form and movement imposed upon the amoebae by systematically changing the pressure-temperature conditions appear to be determined by the effects of temperature and pressure upon the structural state of the plasmagel system. Before discussing this, however, the contradictory evidence of Thornton ('32) in regard to the effect of temperature upon the gel system, should be considered.

Thornton's results, which indicate that the plasmagel displays a firmer structure at low temperature, are difficult to understand on theoretical grounds. Considerable evidence is now available showing that the plasmagel of the amoeba (and all other cells that have been studied) conforms to the Freundlich criteria for a type II system (see Marsland and Brown, '36, and '42; and Marsland, '50). Such gels, by virtue of the volume increase ($+\Delta V$) which occurs during setting, undergo solation when exposed to high pressure; and because

of the endothermic nature of the setting reaction, such gels should be weakened when the temperature is lowered.

The discrepancy between the present observations and those of Thornton, perhaps may be resolved by considering the sources of error which seem to have been present in the earlier work. In the present experiments the centrifugations were performed inside of the thermally controlled chamber, whereas the earlier centrifugations were at room temperature, which must have permitted a thermal drift during the measurements. Also Thornton endeavored to judge the displacement of granules from the plasmagel by studying the living specimens after removal from the centrifuge tubes. In our experience, this procedure does not allow for accurate judgment, since the amoebae very quickly resume an active locomotion, which redistributes the granules before the stratification can be studied very carefully. In our experiments, therefore, the amoebae were subjected to heat fixation (by dropping the centrifuge head into boiling water) within 5 seconds after stopping the spin. Thus ample time was available for a critical analysis of the stratification.

In making a critical analysis of the centrifugal displacement under various conditions, very careful attention must be given to the finer granules, which are last to be displaced from the developing hyaline zone. In figure 4, for example, specimen A might be judged to represent the end-point (actually displayed by B), if one did not take very careful note of the finer granules in specimens centrifuged at higher temperatures (20° and 25°C.), presumably because, as was shown by Mast and Prosser ('32), the thickness of the plasmagel layer (gel/sol ratio) is less than at lower temperatures; and consequently it is easier at higher temperatures to be premature in judging that the end-point has been reached. In short, on the ground of theoretical expectations and the probable sources of error inherent in the earlier work, and in view of the behavior of the amoebae under low temperature conditions, it seems logical to assume that the present measure-

ments are valid, and that the plasmagel structure does undergo weakening as the temperature decreases.

By and large, the results of the experiments provide strong support for the gel contraction theory of amoeboid movement, as formulated by Mast ('26). Accordingly, the current observations will be considered in relation to some of the basic assumptions of the theory. The experiments seem to provide considerable evidence in support of the basic assumption that a gradient exists in the plasmagel system such that newly formed parts, out near the extending tip of a pseudopodium, are generally less firmly gelled and less strongly contractile than the older parts, further back from the tip. The weakest and most labile part of the plasmagel system, in fact, appears to be the plasmagel sheet, which frequently ruptures, even under normal conditions, momentarily allowing the granules of the plasmasol to burst forth and make direct contact with the cell membrane at the tip of the pseudopodium. Consequently, it is not surprising to note that under the solating action of high pressure the plasmagel sheet is first to go, so that it is seen only rarely and transiently even at relatively low pressures (1000–1500 lbs./in.^{2}). Moreover, the side walls of the pseudopodia out near the tip display considerable weakness even at these low pressures, since lateral ruptures, which slightly change the flow direction of the sol, are very frequently observed.

At higher pressures the collapsing of the pseudopodia and the rounding of the specimens provide further evidence of the gradient. Apparently pressure shifts the equilibrium of the whole plasmagel system in the sol direction, but the distal part of the pseudopodium, being less firmly set, reaches a fluid state at relatively moderate pressures, and thus becomes rounded and bulbous first as the pressure level is raised. And as the pressure continues to climb, more and more of the pseudopodia becomes involved, until finally the whole amoeba is converted to a motionless sphere. This indicates that the posterior part of the plasmagel system represents a region where gelation is at a maximum. Moreover, since a similar

series of events is observed at relatively lower levels of pressure when the experiment is done at lower temperature, it may be assumed that low temperature shifts the equilibrium of the whole system in the sol direction, — as is verified by the centrifugal measurements.

The data are likewise consistent with the assumption that the flowing of the plasmasol results from a contraction of the plasmagel, particularly in the posterior lateral parts of this tubular system, where the gel strength is at a maximum. Except for a slight initial lengthening of the pseudopodia, which probably represents a release from the restraining influence of the plasmagel sheet, the protoplasmic flow becomes weaker and weaker, and the diameter and the length of the pseudopodia becomes less and less as the gel structure is weakened by the pressure-temperature conditions. Moreover, it seems evident that the form of the pseudopodium is maintained by the gel structure of the lateral walls, since the pseudopodium rounds up and behaves like a fluid cylinder whenever the pressure-temperature induced solation reaches a critical degree. From this viewpoint, the observation of Mast and Prosser ('32) that the relative thickness of the plasmagel layer (gel/sol ratio) increases at lower temperatures can be regarded as a mechanism which compensates for the weakening of the pseudopodial wall.

A priori, the development of contractility in a fluid system such as protoplasm would seem to presuppose the formation of some kind of gel structure. If, as seems likely, the contractile force originates from a folding of elongate protein molecules or molecular aggregates, it is difficult to see how such a folding could be effective in performing work unless the extended molecular units were interlinked, forming a continuous and fairly extensive system throughout the cell. In fact, as Kopac ('50) states, it is generally agreed that gelation requires the formation of three-dimensional networks from fibrillar units present in the system (see also Ferry, '48). Therefore, it is not surprising to find that contractile parts of the protoplasm must display a certain minimum of

gel structure, and that the magnitude of such contractile forces in the protoplasm is limited by the tenacity of the intermolecular linkages of the gel structure.

The gel contraction theory likewise provides an explanation for the "decompression contraction phenomenon," which is shown in figure I, D. The inert spherical form of the amoeba observed at high pressures represents, apparently, a complete solation of the plasmagel, and the complete disappearance of any orientation or gelational gradient in the system. When suddenly released from the pressure constraint, apparently, all of the peripheral part of the cytoplasm becomes strongly and uniformly gelled. Then there is a strong generalized contraction accompanied by a detachment of the cell membrane from the subjacent plasmagel all over the cell surface.

The contraction of the gel, apparently, involves the squeezing forth of a fluid component which initially is enmeshed in the colloidal three-dimensional network of the gel structure —or otherwise the network proper could not display an appreciable degree of shrinkage. Necessarily the expressed fluid must be hyaline, since visible granules are too coarse to escape from the colloidal network. Ordinarily the escape of hyaline fluid from the contracting plasmagel cannot be seen, except perhaps locally in the hyaline cap of an advancing pseudopodium, since elsewhere a firm attachment of the cell membrane to the subjacent gel prevents the fluid from escaping peripherally — and centrally the hyaline fluid quickly intermingles with granular flowing plasmasol. Probably not all of the fluid in the peripheral hyaline zone which develops after decompression is derived from the interstices of the gel structure, since the plasmagel layer also seems to act as a sieve which permits the egress of fluid, but not of granules, from the deeper-lying plasmasol. In any event, the simplest interpretation of the decompression reaction seems to be that it represents a strong generalized contraction of the plasmagel system. Temporarily the system appears to lack any kind of oriented organization or gradient, and normal locomotion

is not resumed until such an organization has been re-established.

The endothermic nature of protoplasmic gelation reactions presupposes that energy from some metabolic source must be utilized in the setting of the plasmagel system. The limiting factor in the present experiments, however, appears to be a direct action of pressure and temperature upon the gel structure per se. In other words, the experiments give little information in regard to the pattern of metabolism which must determine the cyclic and oriented reactions of gelation and solation which occur during normal locomotion. Some phases of this metabolism, on the other hand, appear to continue even at pressures which completely inhibit gelation — since the exceptionally strong contraction, which occurs immediately after decompression, seems to indicate an accumulation of one or more critical metabolites during the compression period.

As reported by Marsland and Brown ('42), the adenosine triphosphate (ATP) system appears to be active in the in vitro gelations of freshly prepared "myosin" sols; and it seems reasonable to postulate that an ATP system likewise donates energy to the plasmagel system of the amoeba. Recently, in fact, this hypothesis has received strong support from several quarters. Kriszat ('49) has reported an increase in the cytoplasmic viscosity of Chaos chaos after treatment with ATP. Loewy ('52) has extracted an actomyosin-like protein from the plasmodium of a Myxomycete and shown that the structural state of such extracts displays changes when ATP and allied comounds are added to the system. Also Hoffmann-Berling and Weber ('53) have demonstrated that a drastic contraction occurs in glycerol extracted fibroblasts when ATP is added and that this contraction can be stopped and started again by the addition and subtraction of ATP-ase inhibitors. And finally, the work of Marsland, Landau and Zimmerman ('53) indicates that ATP can contribute energy to the plasmagel system of dividing Arbacia eggs, improving their furrowing potency. Accordingly, fur-

ther experiments, designed to test this hypothesis in relation to the form and movement of Amoeba proteus, are now in progress.

SUMMARY

Centrifugal measurements of the structural state of the plasmagel layer of the amoeba, made at two temperatures (15° and 25°C.) and at various pressures (up to 10,000 lbs./in.^{2}), show that the sol $\rightleftarrows$ gel equilibrium is shifted in the sol direction by decreasing temperature, as well as by increasing pressure. Thus temperature and pressure are found to have opposite effects upon the equilibrium, each temperature change of 5° being approximately equivalent in action to a pressure change of 1000 lbs./in.^{2}.

The behavior of the amoebae, observed directly under the various temperature-pressure conditions, seem clearly related to the structural state of the plasmagel system. Whenever the gel strength of this part of the protoplasm is reduced below a critical value, by low temperature or high pressure, acting separately or in combination, the pseudopodia undergo collapse and the cell as a whole rounds up, apparently as a result of tensional forces at the cell surface. Moreover, if the gel system is weakened in lesser degree, a corresponding decrease in the size of the pseudopodia can be observed.

In general, the experiments provide further evidence as to the endothermic character of protoplasmic gelations, and substantiate the view that gel structures, by virtue of their contractile properties, are instrumental in transforming metabolic energy into mechanical work.

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THE EFFECT OF DEHYDRATION AND LOW TEMPERATURE ON RENAL FUNCTION IN THE BULLFROG

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FIVE FIGURES

The frog, like most other amphibians, is able to migrate from water to dry land and vice versa. In doing this, changes occur in its water balance. When the frog is in water it will gain water by diffusion through the skin. The rate of uptake varies with the temperature and is 4–5 times as rapid at 30°C. as at 0°C. (Overton, '04; Hevesy, Hofer and Krogh, '35). When the frog is out of water, water evaporates freely from the surface of the skin at a high rate; and Overton found that a 60 gm frog loses one-quarter of its weight in 8–11 hours at ordinary room temperature and humidity (quoted from Krogh, '39).

The frog responds physiologically to changes in its environment in such a way that it tends to maintain the water content of the body. When the frog is in water the kidneys usually eliminate water at the same rate with which it is taken up. The rate of urine production therefore varies with the temperature. When out of water the urine flow decreases.

The purpose of the present study has been to investigate the changes in renal plasma flow, glomerular filtration rate and tubular reabsorption of water that are responsible for the changes in urine flow when the frog is exposed to changes in its environment.

It was also decided to study the urea excretion. This is of particular interest in the frog because it has been suggested by Marshall ('32) and later by Walker and Hudson ('37) that this animal is able to excrete urea by renal tubular secretion. A comparison between the urea clearance and the simultaneous PAH clearance would show whether the former is so close to the PAH clearance that it might give a measure of the renal blood flow.

Another reason for studying the urea clearance is that it has been suggested that certain mammals, the rodents *Heteromyidae* (Schmidt-Nielsen, in press), also can excrete urea by renal tubular secretion but that this excretion is easily depressed by excitement and stress. It was of interest to determine whether these factors also could affect the urea secretion in the frog.

METHODS

Creatinine clearances were used to measure glomerular filtration rates (Forster, '38) and p-aminohippurate (PAH) clearances for renal plasma flow determinations. PAH clearances have been found to be identical with diodrast clearances (Forster, '43). The bullfrogs *(Rana clamitans)* were taken from local ponds (Mt. Desert Island, Maine) and used for experiments within a day or two of capture. Body weights (BW) varied from 100 to 170 gm. (It was noticed that this species of bullfrog, which is smaller than the Louisiana bullfrog (Rana catesbiana), also showed a somewhat different behavior in the laboratory. While *Rana catesbiana* spends a considerable part of its time completely submerged in water, the species we worked with always kept its head over water at ordinary room temperature. It is perfectly possible that some physiological responses differ in the two varieties. A few experiments on Louisiana bullfrogs showed that they do not respond similarly to blood sampling, but their response to a dry environment was the same as that found in the Mount Desert Island bullfrogs.)

When PAH and creatinine clearances were studied simultaneously, a priming dose of 1 to 6 mg of PAH and 10 mg

of creatinine per 100 gm BW was injected into the dorsal lymph sac at the start of the experiment. Blood levels were sustained by hourly maintenance doses of 0.6 mg PAH and 1 mg creatinine per 100 gm BW. The urine samples were collected every hour by means of a specially designed glass catheter (Forster, '38). Blood was withdrawn directly from the heart. Usually one sample was taken early in the experiment and one at the termination of the experiment. To test the effect of a dry environment, frogs, after being kept in water for 4 control clearance periods, were then placed in an open wire cage for varying periods of time and after this again returned to water. The effect of water diuresis was tested in frogs that were injected with distilled water in an amount corresponding to 15% of their body weight. The water was injected into the dorsal lymph sac. These frogs were kept in water throughout the experiment.

In the "cold" experiments only filtration rates and urea clearance were determined. Creatinine was administered in a single injection. The following day the frogs were acclimatized to low temperature by being kept in water at 2 to 5°C. for 24 hours; then the cloaca was closed with a ligature. This was necessary because the urine would otherwise leak out, due to the pressure from the enormously increased amount of fluid in the peritoneal cavity of these "cold" frogs. Five hours later the urine was collected, and a blood sample was taken by heart puncture.

Creatinine was determined using Clark and Thompson's modification ('49) of the Folin and Wu method. Urea was determined by Conway's microdiffusion method ('42), and PAH by Bratton and Marshall's method ('39).

RESULTS AND DISCUSSION

Effect of a dry environment

Some of the individual responses are presented in graphical form in figures 1, 2 and 3. Despite a considerable variation between the individual frogs, with respect to the magnitude

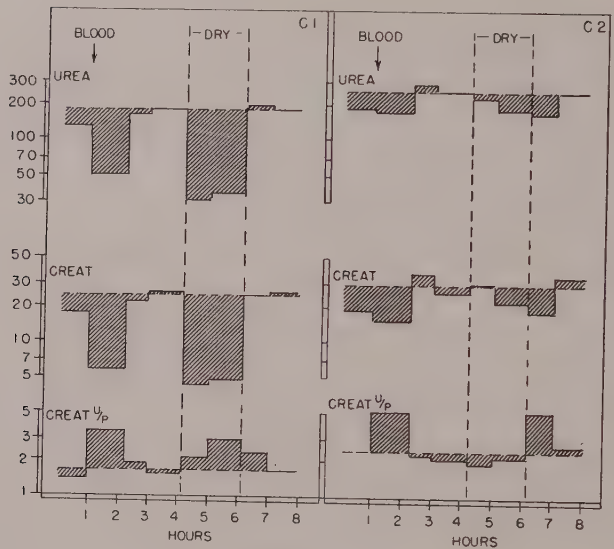

Figure 1

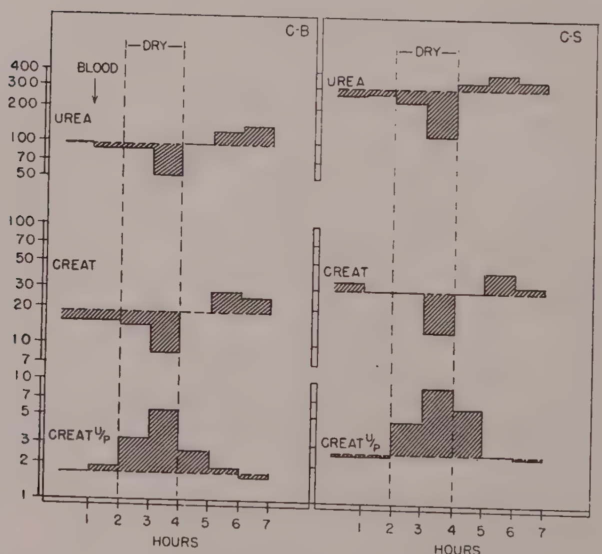

Figure 2

Figs. 1, 2 and 3 Individual responses to a period out of water.

Ordinate: The two upper scales give clearances in ml/kg hr. The lower scale in figures 1 and 2 give creatinine U/P ratios. In figure 3 the lowest scale gives the filtration fraction (creatinine/PAH clearance ratio).

Abscissa: Hours from start of urine collections.

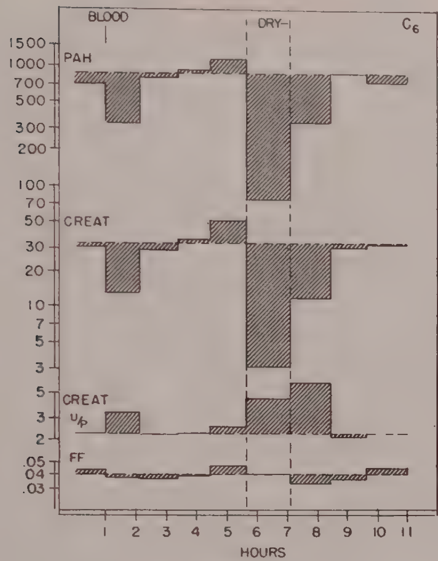

Figure 3

TABLE 1

Effects of dehydration, water injection and low temperature

	URINE FLOW	CREATININE U/P	FILTRATION RATE	UREA/ CREATININE CLEARANCE RATIO	NUMBER OF FROGS
	ml/kg hr.		*ml/kg hr.*		
In water ''normal'' 20–25°C.	13.2	2.69	34.2	6.5	6
First hour out of water	7.10	5.82	28.1	6.2	6
Second hour out of water	2.98	10.03	19.0	6.45	6
Fifth hour out of water	0.84	6.52	5.1	3.4	2
In water 20–25°C. water injection	52.8	1.56	82.0	6.3	2
In cold water 5°C.	3.16	1.54	4.5	5.0	7

The figures presented in this table are average values from a number of frogs. The first three figures in each column represent the same 6 frogs during three consecutive hourly clearance periods, viz.: (1) the last period in water before the animals were transferred to a dry cage, (2) the first period out of water, and (3) the second period out of water.

of the response and the time at which it set in, the general trend is clear. This is seen from table 1, where the average values are presented.

The effects upon renal function of exposure to a dry environment at room temperature are: (1) an increased tubular reabsorption of water (increased creatinine U/P ratios) and (2) decreased glomerular filtration rate. During the first hour of dehydration the decrease in filtration rate is only moderate, but already during the second hour the filtration rate has fallen about 50%; and if the frogs are kept in a dry environment for as long as 5 hours the filtration rate drops to an average of 15% of what it is when the frogs sit in water at the same temperature. The tubular reabsorption of water, which is increased 4-fold during the second hour of dehydration, is not so much increased in the later stages of dehydration. After 5 hours of dehydration the frogs had lost on the average 14% of their body weight and the plasma electrolyte concentration was increased from 111 mM to 125 mM. The frogs injected with water showed a marked increase in diuresis caused by an increased filtration rate and a decreased reabsorption of water. The plasma electrolyte concentration was decreased to 97.5 mM.

Sawyer ('51) has found that the oxytocic fraction from the mammalian posterior pituitary, which is responsible for the "water balance effect" in the frog, also exerts an effect upon the kidneys. Thus, he has found that the urine flow decreases when the hormone is injected into frogs. He found that the decrease in urine flow is proportional to the decrease in filtration rate. He therefore concluded that the rate of urine formation is controlled chiefly by changes in filtration rate and that the pituitary hormone has a glomerular, but no tubular, effect upon the kidneys of the frog. Since the neurohypophysis in the frog becomes increasingly depleted of hormones during dehydration (Levinsky and Sawyer, '53) and since the effect of these hormones are similar to the effects of dehydration it is most likely that the responses to dehydration are connected with the release of posterior

pituitary hormone. However, there is a discrepancy between our findings with dehydration and Sawyer's with hormone injections in that we found a tubular, as well as glomerular, regulation of the urine flow, while Sawyer found only a glomerular effect.

Renal responses to blood sampling were observed in almost every frog. As mentioned under methods, a blood sample was taken by heart puncture in the preliminary period

TABLE 2

Effects of blood sampling

FROG NO.	URINE FLOW		CREATININE U/P		FILTRATION RATE		UREA/CREATININE CLEARANCE RATIO	
	Before	After	Before	After	Before	After	Before	After
	ml/kg hr.				*ml/kg hr.*			
A-R	24.0	22.6	1.30	1.46	31.2	32.9	6.0	5.5
A-L	28.8	13.2	1.45	2.58	41.7	34.1	6.5	5.5
C-B	9.3	8.4	1.65	1.87	15.4	15.7	6.2	6.6
C-1	12.6	1.7	1.38	3.47	17.4	5.9	7.5	8.5
C-2	7.9	2.9	2.41	5.38	19.1	15.6	9.1	11.7
C-6	13.7	3.9	2.28	3.28	31.2	13.8		
C-7	20.8	5.6	1.52	3.30	31.6	18.5	2.8	3.3
C 8	13.5	5.7	2.58	4.97	34.9	28.4	5.7	6.5
C 9	21.5	8.7	2.66	5.05	57.2	43.9	10.2	10.6
Average	16.9	8.1	1.91	3.47	31.1	23.1	6.7	7.1

The values presented in the columns marked "Before" are the values obtained in the clearance period directly preceding the blood sample. The values presented in the columns marked "After" are the values obtained in the clearance period directly following the blood sample.

(frogs sitting in water). Such responses usually did not last for more than one hour (figs. 1 and 3). From table 2 it can be seen that the responses, which are similar to the effect of a short period out of water, are an increased tubular reabsorption of water and a decreased filtration rate. The effect might be due to the sudden decrease in blood volume, but could also be a result of shock or stress.

Effect of a cold environment

Bullfrogs transferred suddenly from water of room temperature to water of 2°–5°C. submerge, arrest respiratory movements and remain motionless at the bottom. Little or no urine is formed for the next 12–18 hours. During this period the body weight increases some 5–6%. After this quiescent period urine flow again commences, but at a lower rate, and the body weight remains fairly constant. Barker-Jorgensen ('50) has shown that the water retention is not secondary to an active retention of salt since the increase takes place also in distilled water. The frogs actually become

TABLE 3

Effects of low temperature

FROG NO.	URINE FLOW	CREATININE U/P	CREATININE CLEARANCE	UREA/CREATININE CLEARANCE RATIO
	ml/kg hr.		*ml/kg hr.*	
1	2.89	1.18	3.42	3.2
2	1.53	2.53	3.86	8.1
3	5.00	1.38	6.90	5.9
4	2.03	1.63	3.30	7.1
5	4.10	1.02	4.15	4.1
6	2.90	1.86	5.40	3.4
7	3.66	1.15	4.20	3.4
Average	3.16	1.54	4.47	5.0

osmotically diluted by the retention of water. In our experimental frogs, acclimatized to 5°C., the electrolyte concentration of the plasma averaged 90 mM against the normal concentration of 111 mM. When the frog is warmed after having been cold the water is again excreted. The retention of water also takes place when the frog is cooled slowly in its natural habitat in fall and winter.

It was not possible for us to follow the renal function in the frogs during the period of acclimatization to cold because no urine could be obtained. After the frogs were acclimatized, and urine was formed at a low rate, clearance determinations were made. The individual results are presented in table 3.

It is seen that the urine flow is about one-quarter of the flow at 20–25°C. (table 1). The tubular reabsorption of water is very low, and the filtration rate is reduced to less than one-seventh of the rate at the higher temperature. The decreased filtration rate is therefore solely responsible for the low urine flow. The renal plasma flow could not be determined in these experiments, but judging from the urea/creatinine clearance ratio it can be assumed that also the renal plasma flow was reduced at the low temperature.

Renal plasma flow, filtration fraction and p-aminohippurate Tm

The renal plasma flow was determined in 6 frogs, two of these had induced water diuresis. The other 4 were used in dehydration experiments, thus they were out of water for a short period each, but were in water most of the time. The filtration rates varied from 3 to 57 ml/kg hr. in the 41 clearance periods from these frogs, but the filtration fraction (FF) varied only about 20%. The average FF was found to be 0.0412, standard deviation: 0.0086. Figure 3 illustrates the variations in renal plasma flow during a dehydration experiment and shows the constancy of the filtration fraction, despite wide variations in glomerular filtration rate.

In 6 other frogs the plasma concentrations of PAH were too high to permit all of the PAH carried to the tubules to be excreted because the maximum capacity of the tubules (Tm) was reached.

The Tm_{PAH} values were calculated in these experiments.[3] In figure 4, Tm values for two frogs are plotted against filtration rates. It is seen that Tm_{PAH} is proportional to the filtration rate. Similar relationships were found in the other 4 frogs. This finding indicates that the nephrons work intermittently, i.e., at lower filtration rates a smaller number of nephrons are functioning than at higher filtration rates. The

[3] In calculating the Tm_{PAH} no correction for protein-bound PAH was made. The value for FW has never been determined in the frog.

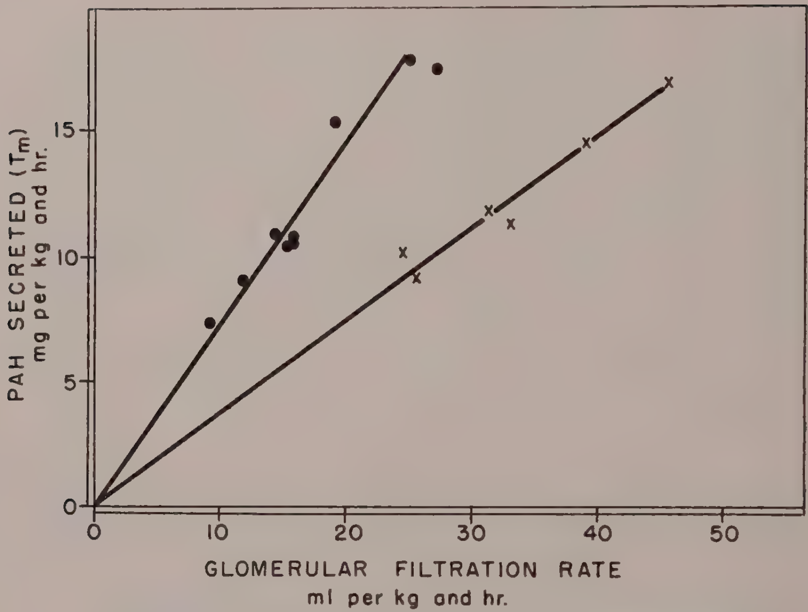

Fig. 4 Relationship between the maximum amount of p-aminohippurate secreted and the glomerular filtration rate. The data represent two frogs.

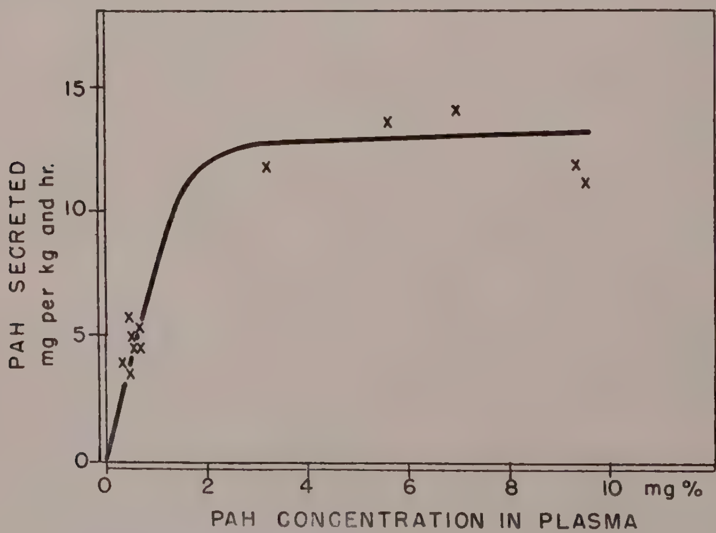

Fig. 5 Relationship between the amount of p-aminohippurate secreted and the concentration in the plasma. The data are from 8 frogs. Clearance periods with a glomerular filtration rate between 31 and 34 ml/kg hr. have been used.

same deduction was made earlier (Forster, '42) from the finding that the amount of glucose reabsorbed in the tubules of the frog per unit time varies directly with the concurrent rate of filtration.

In figure 5 the amount of PAH secreted has been plotted against the plasma concentration. Values with a filtration rate between 30 and 34 ml/kg hr. have been used. The curve shows that at a constant filtration rate the PAH secretion is limited by a tubular maximum as in other animals.

An attempt to calculate an average Tm value was made by using 20 clearance periods with filtration rates from 20–40 ml/kg hr. (average 30.4). The Tm was found to be 14.3 mg PAH/kg hr. The average renal plasma flow is 775 ml/kg hr. when the filtration rate is 30.4 ml/kg hr. This means that on an average the maximum capacity of the tubules is reached when the plasma concentration of p-aminohippurate is 1.8 mg %.

Urea excretion

The urea clearance was consistently higher than the simultaneous glomerular filtration rate. In 87 clearance periods on 12 frogs the mean urea/creatinine clearance ratio was 6.9 ± 0.3 with a standard deviation of 2.3. This is in accord with the previous findings by Marshall ('32) and Walker and Hudson ('37) that urea is actively secreted by the tubules of the frog. The urea clearance was consistently lower than the renal plasma flow. The average RPF/urea clearance ratio being 3.4 ± 0.2. Standard deviation 1.0. The reasons for the lower urea clearances might be either that urea, which is a highly diffusible substance, diffuses back to some extent or that the tubular transport of urea is characterized by a low Tm so that the transport mechanism is saturated already at the normal plasma concentration of urea. If the latter were the case the urea clearances should increase with decreasing plasma concentration of urea (provided the filtration rate was constant). If the lower urea clearances were caused by back diffusion of urea we could expect to find that the urea clearances would decrease with

increasing tubular reabsorption of water. Clearance periods with filtration rates around 30 ml/kg hr. have been listed in table 4. It appears that there is some increase in urea/creatinine clearance ratio with decreasing plasma urea con-

TABLE 4

Urea excretion

FROG NO.	CREATININE CLEARANCE	PLASMA UREA CONC.	CREATININE U/P	UREA/CREATININE CLEARANCE RATIO
	ml/kg hr.	*mM*		
C-9	30.0	0.48	2.72	8.5
C-9	35.4	0.49	2.07	10.4
C-2	37.8	0.55	2.28	7.8
C-2	30.8	0.56	1.95	7.4
C-2	35.8	0.57	2.76	7.0
C-S	33.9	0.75	2.63	10.7
C-S	29.6	0.75	2.50	9.1
C-S	35.0	0.75	2.50	7.5
C-S	27.4	0.75	2.92	9.8
A-S	34.5	0.76	20.2	4.1
A-S	27.7	0.76	1.84	3.8
A-R	31.2	0.94	1.30	6.1
A-R	32.9	0.98	1.46	5.4
A-B	30.4	1.00	19.8	5.1
A-B	36.4	1.00	2.50	4.9
C-8	28.4	1.05	4.97	6.5
A-R	25.5	1.07	1.32	5.0
C-8	34.9	1.09	2.58	5.7
C-6	31.2	1.45	2.28	6.7
C-1	25.8	1.52	1.67	7.3
C-1	26.3	1.58	1.51	6.9
C-B	27.7	1.84	1.82	4.3
A-L	34.1	3.07	2.58	5.5

centration. It is surprising to find that a high tubular reabsorption of water (creatinine U/P ratio: 20) does not lower the urea/creatinine clearance ratio. From this it seems possible that the urea excretion is limited by a low Tm rather than by back diffusion of urea in the tubules. However, the material is much too limited to reach any definite conclusion on this point.

Tubular secretion of urea was maintained under all of our experimental conditions. Excitement or stress as caused by handling, blood sampling, or dehydration (tables 1, 2 and 3) did not have any selective effect upon the urea excretion.

SUMMARY

A frog exposed to a dry environment reduces its urine flow by reducing the filtration rate and increasing the tubular reabsorption of water. The reduction in glomerular filtration rate is accompanied by a parallel reduction in renal plasma flow. When the frog is exposed to prolonged dehydration the decrease in filtration rate becomes more pronounced, and the increase in tubular reabsorption of water less pronounced. The antidiuretic response therefore, particularly in the earlier stages of dehydration, appears to be a tubular as well as a glomerular response.

The renal response to a cold environment diffffered from the response to dehydration in that the tubular reabsorption of water was very low, lower than under "normal" conditions. The glomerular filtration rate was considerably reduced.

Studies of the excretion of p-aminohippurate showed that the Tm value for this substance is directly proportional to the filtration rate. This is taken as supporting evidence for an intermittent function of the nephrons, i.e., the number of functioning nephrons is proportional to the filtration rate. The average Tm_{PAH} value was determined to be 14.3 mg/kg hr. when the filtration rate is 30 ml/kg hr.

Studies of the excretion of urea showed that the urea clearances are on the average 7 times higher than the concurrent filtration rates, but that they are exceeded about three and a half times by the renal plasma flows. The problem of determining whether urea clearances are lower than PAH clearances because of back diffusion of urea subsequent to secretion or because its transfer process is relatively sluggish could not be determined. The urea/creatinine clearance ratio did not vary significantly under the different

experimental conditions, and it seems that the tubular secretion of urea in the frog is unaffected by stress.

ACKNOWLEDGMENTS

These studies were aided by a research grant from the Office of Naval Research (B. S-N.) and by a grant from the Rockefeller Foundation (R. P. F.). Mr. Willian Barkley contributed valuable technical assistance.

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ACTIVATION OF A NON-PROPAGATING MUSCLE IN THYONE [1]

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FIVE FIGURES

Some short-fibered non-striated muscles show propagated action potentials, as the Phascolosoma probiscis retractors and the Thyone lantern retractors; others fail to propagate, as the long body wall retractors of Thyone (Prosser, Curtis and Travis, '51). In the non-propagating muscles, contraction occurs only in the region of stimulation. Propagation in the Phascolosoma proboscis retractors is by intrinsic nerve fibers which appear to activate many muscle fibers successively (Prosser and Melton, '54). The present paper deals with the innervation of a non-propagating smooth muscle, the long body wall retractors of *Thyone briareus* (Lesueur).

MATERIALS AND METHODS

Freshly collected sea cucumbers were used, and the muscles were dissected in either natural or artificial sea water. Stimulation and recording was by chlorided silver electrodes making contact by means of wicks under light mineral oil. Action potentials were obtained by a direct-coupled amplifier, contractions by an RCA 5734 transducer; both were recorded on a Grass inkwriter. Artificial sea water was made by the formula of Hodgkin and Katz, '49.

[1] Work described in this paper was in part supported by contract between the Medical Division, Chemical Corps, U. S. Army and the University of Illinois. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions and conclusions of the author.

[2] I am indebted to Charles E. Smith, Jr. and Ronald A. Bergman for assistance in histology and photography.

RESULTS

Action potentials. When carefully dissected, the long retractors of Thyone invariably shorten by at least one-third and remain in this state. It is very difficult to obtain any electrical responses from such muscles. If, however, a muscle is stretched to its original extended length, action potentials can be recorded close to the stimulating cathode. Often it is necessary, even with extended muscles, to move the wick leads about extensively before one obtains a good record. This muscle has much connective tissue and relatively sparse muscle fibers, and it appears that good contact with a number of muscle fibers is difficult to obtain. Attempts to record by silver wires inserted into the muscle were unsuccessful.

The recorded action potentials have a distinct threshold but increase to a maximum with increasing intensity (fig. 1).

As the separation between stimulating and proximal recording electrodes is increased the amplitude of recorded potential is reduced. Action potentials can usually be detected out to 8 and sometimes to 12 mm from the point of stimulation. This distance makes it unlikely that the potentials are electrotonic in nature. Reduction in amplitude varies with contact but the reduction is approximately by two-thirds on going from 2 to 10 mm distance from the stimulated region. Also conduction is at a measurable velocity. When latencies are compared over the distance of recording the velocity is on the average 16.7 cm/sec. (limits 13 to 20.8 cm/sec. at 23–25°C.) (fig. 2).

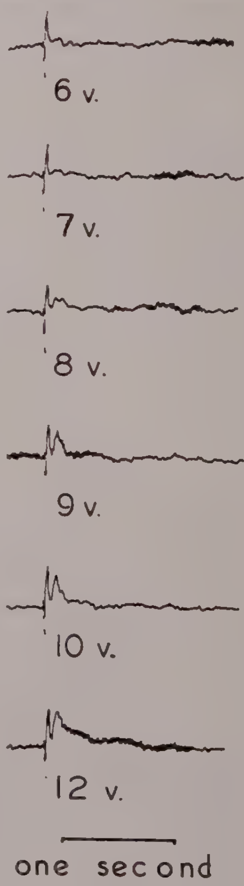

Fig. 1 Responses to single shocks at increasing intensities, 6 to 12 volts; records show threshold and gradation of response.

On repetitive stimulation the amplitude declines much as in the fast response of the Phascolosoma retractors. At a frequency of 0.5/sec. the amplitude is constant or declines very slightly, at 1/sec. the response falls off after two or three stimulations and at two or three per second decline in amplitude to near zero occurs after one response (fig. 3).

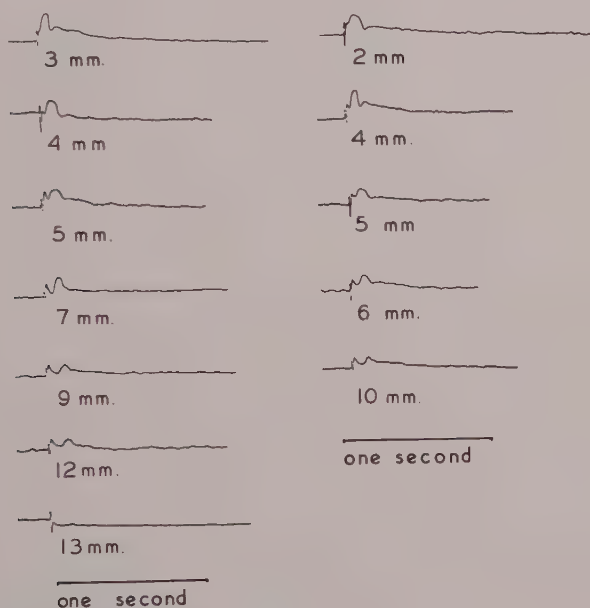

Fig. 2 Responses at different conduction distances in two different experiments; records show decline in amplitude and increase in latency with increasing distance.

When an excised long retractor of Thyone is soaked in a solution of eserine in artificial sea water for 15 to 30 minutes, responsiveness is lost at concentrations of 10^{-6} and higher; however at 10^{-7} and 10^{-8} the response to a submaximal shock is greatly enhanced above that in sea water (fig. 4). Also local application of eserine at 10^{-6} facilitated the response whereas higher concentrations blocked.

Contraction records show that the muscle remains contracted long after the action potential is completed. Contrac-

tion times for single twitches were 0.5–1 sec. and half-relaxation times varied from 2 to 8 seconds (fig. 3).

It is concluded that in extended long retractors of Thyone an action potential is detected at declining amplitude out to some 12 mm from the stimulus; this response is graded, fatigues rapidly on repetition at 1/second, is conducted at about 17 cm/sec. and is enhanced by eserine.

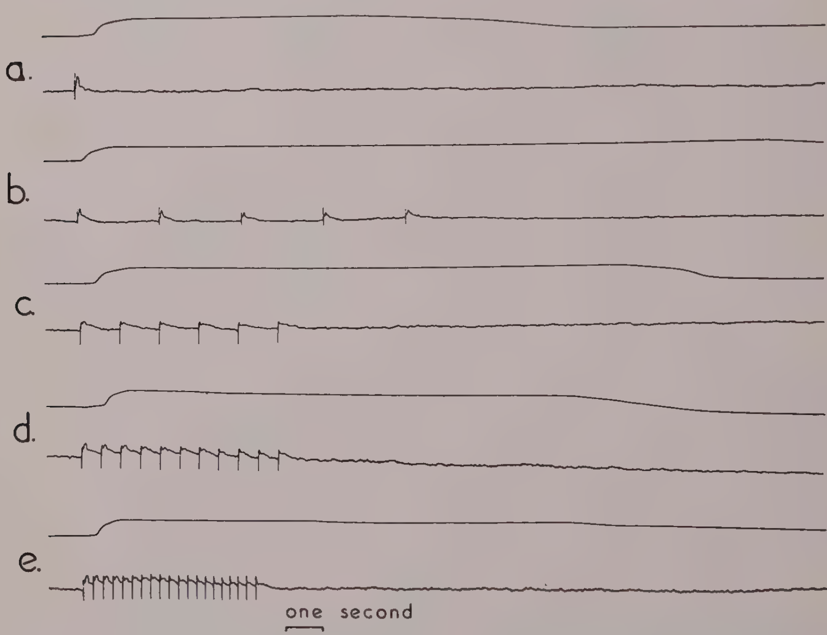

Fig. 3 Simultaneous contraction and action potential records. Frequencies: a single shock, b 0.5/sec., c 1/sec., d 2/sec., e 4/sec.

Histology. Attempts to stain nerve fibers on the long retractors by methylene blue were not successful. However when reduced methylene blue or rongalit, prepared as directed by Smith ('46) for nerves in starfish was used, there was some staining of nerve elements. Fine blue nerves could be seen extending out, usually accompanied by haemal vessels, from the body wall to the long retractors. These nerves could be seen only when the body wall was separated from the muscle by

traction and the nerves were so fragile that they often broke when stretched. Photography was difficult because of the irregular thickness but nerves are indicated in figure 5. The nerves were present at intervals of slightly less than 1 mm.

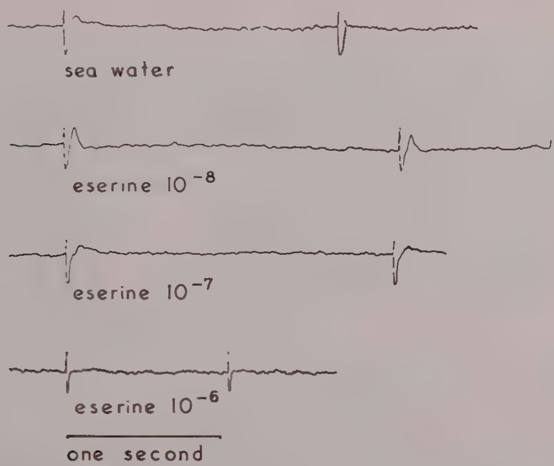

Fig. 4 Effects of soaking muscle for 15 to 30 minutes in sea water, eserine 10^{-8} (marked potentiation), eserine 10^{-7} (slight potentiation), eserine 10^{-6} (depression). Stimulus intensity and electrode positions uniform.

Sections of the body wall and attached muscle show a radial nerve within the body wall below each long retractor muscle. These radial nerves give off frequent lateral branches which pass at various angles to the long muscles (fig. 5).

Macerations in acid show that the muscle fibers of the long retractors are narrower and longer than those of the Phascolosoma proboscis retractor. They measured approximately 2 μ wide and 1 to 1.2 mm long.

DISCUSSION

The preceding results indicate that impulses are conducted at decreasing amplitudes and at a velocity of about 17 cm/sec. for short distances in the long retractors of Thyone. Potentiation by eserine indicates that the innervation may be cholinergic; the corresponding muscles of Stichopus are used in the

bioassay of acetylcholine (Bacq, '39). The explanation of the failure to propagate for the full length of the muscle lies in the successive local innervation of the muscle by many branches from the radial nerve. Fibers in these branch nerves must extend 5 to 12 mm in the muscle and must overlap one another.

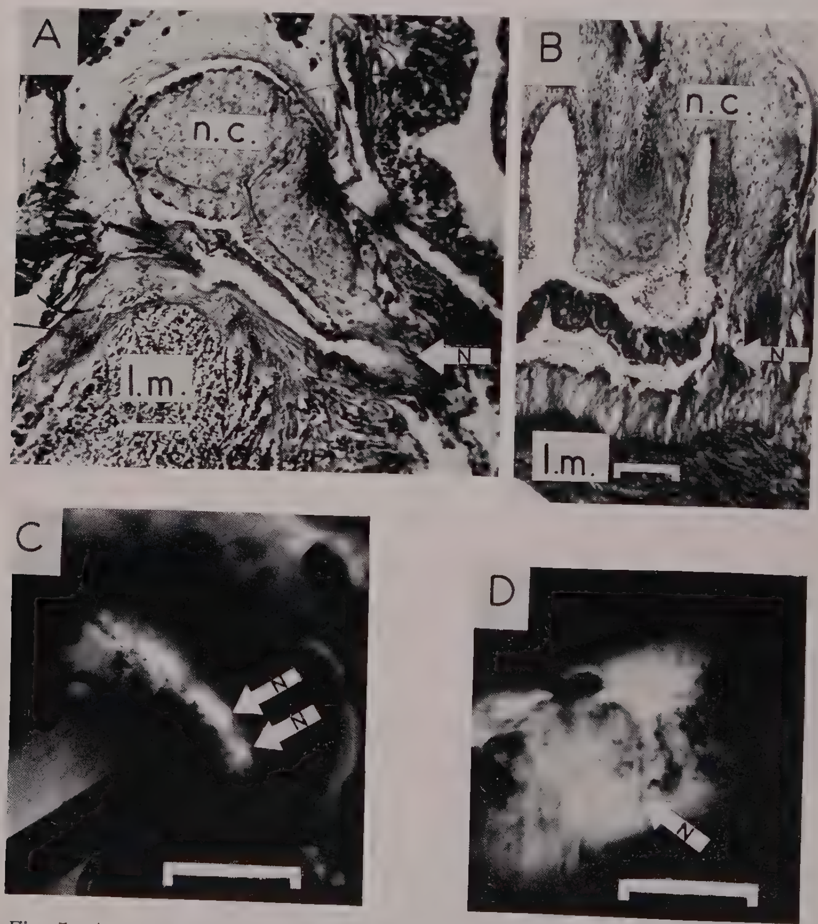

Fig. 5 A cross section, B longitudinal section through part of body wall and long retractor muscle. n.c. radial nerve, N. nerve branch to muscle, l.m. long retractor muscle. C, D photographs of living mounts of thick pieces of body wall separated by traction from the long retractor muscles; arrows point to the thin nerve branches stained with methylene blue. Magnification indicated by brackets: A and B 0.1 mm, C, D 1 mm.

Thus the limited conduction represents the extent of these nerves. Conduction from one end to the other of the intact animal is, therefore, by the radial nerves.

SUMMARY

1. When long retractors of the body wall of Thyone are extended to rest length, action potentials can be obtained near the point of stimulation but there is no propagation from one end of the muscle to the other. The recorded potentials decrease with distance and are barely detectable at 10 to 12 mm.

2. The action potentials decline in amplitude on repetition at 1/sec. but remain nearly constant at 0.5/sec. and lower frequencies.

3. Conduction over the distance from which potentials can be recorded is at 17 cm/sec.

4. Treatment with eserine (10^{-8} to 10^{-7}) enhances the responses to submaximal shocks.

5. Many small nerves pass from the radial nerves to the long retractor muscles.

6. It is concluded that the recorded action potentials represent activity in muscle innervated by short branching nerves.

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NERVOUS CONDUCTION IN SMOOTH MUSCLE OF PHASCOLOSOMA PROBOSCIS RETRACTORS [1]

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THIRTEEN FIGURES

The action potential of proboscis retractors of *Phascolosoma gouldii* (Pourtales) consists of two components (Prosser, Curtis and Travis, '51): (1) an all-or-none fast spike which fatigues rapidly on repetitive stimulation and (2) a graded slow wave which facilitates on repetitive stimulation. The fast wave (velocity 1.35 m./sec. at 23°C.) is associated with a quick phasic contraction, and the slow wave (velocity 0.3 m./sec.) with maintained tonic contraction. The fast wave usually has a lower threshold and is lost sooner than the slow wave on treatment of the muscle with excess potassoum or on degeneration. Histological examination failed to show two types of muscle fiber. The present study was undertaken to learn the basis for the two components of the muscle potential.

MATERIALS AND METHODS

Action potentials were recorded from isolated muscles, usually suspended in paraffin oil, with chlorided silver electrodes making contact through 0.7 mm wicks soaked with

[1] Work described in this paper was in part supported by contract between the Medical Division, Chemical Corps, U. S. Army and the University of Illinois. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions and conclusions of the authors.

[2] We are indebted to Charles E. Smith, Jr., for assistance with the recording equipment and to both Mr. Smith and Ronald A. Bergman for assistance in histology. Present address of C. E. Melton: Physiology Department, Western Reserve University, Cleveland, Ohio.

sea water. When the nerve leading to the ganglion which innervates the retractors was to be stimulated the entire proboscis and one attached retractor were mounted in a paraffin block grooved so that the muscle could be treated separately from the ganglion. Natural sea water was used as the physiological saline in the earlier experiments but artificial sea water made according to the recipe of Hodgkin and Katz ('49) gave longer survival and was used in most experiments. Records with a direct-coupled amplifier were recorded on a Grass ink-writer and with a condenser-coupled

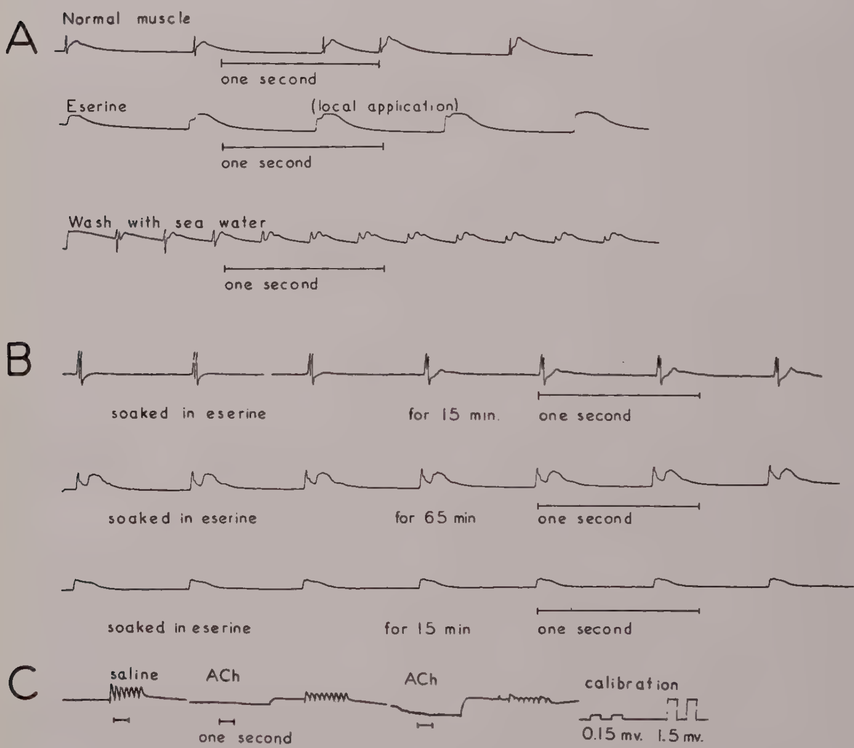

Fig. 1 Effect of eserine and of acetylcholine on action potentials. A. Effect of eserine (1×10^{-5}) applied locally at active recording electrode. B. Effect of soaking muscle in a low concentration (1×10^{-7}) and in a higher concentration (1×10^{-6}) of eserine. C. Effects of local application of a drop of sea water as control, acetylcholine (3×10^{-5}) and slightly higher (2×10^{-5}).

amplifier on a cathode ray oscilloscope. Contractions were measured by means of an electronic transducer tube (RCA 5734) and recorded on a second channel of the ink-writer.

RESULTS

Effects of drugs. The two conduction systems were first differentiated by means of drugs dissolved in artificial sea water.

Physostigmine. When eserine sulphate (10^{-5}) was applied to the muscle the slow wave was markedly enhanced so that the first slow response of a series was nearly maximal (fig. 1). There was also a tendency to repetitive response of both fast and slow components. Muscles soaked for about 15 minutes in eserine (10^{-6}–10^{-7}) showed enhanced responses; at higher concentrations or after prolonged soaking in low concentra-

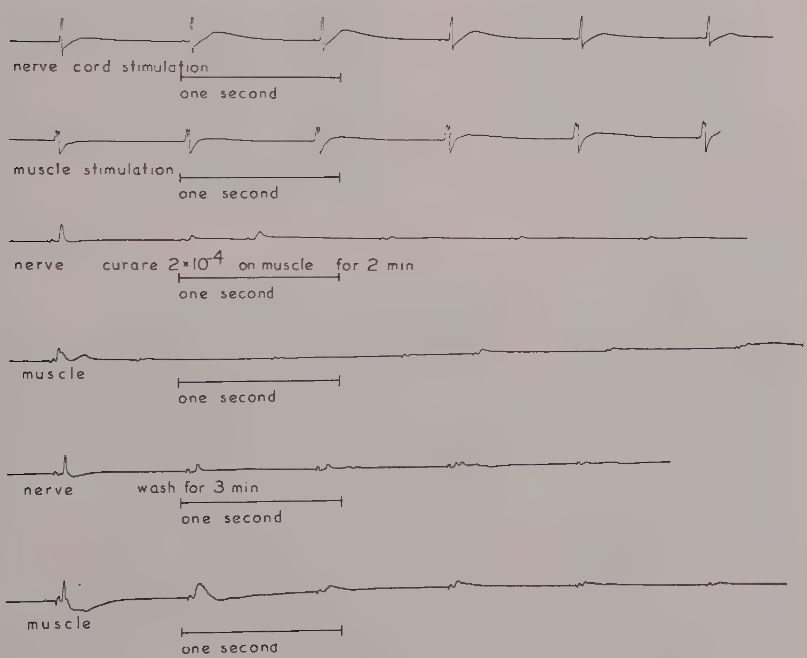

Fig. 2 Electrical responses of muscle to stimulation of nerve cord and of muscle before treatment, after application to muscle of tubocurarine (2×10^{-4}) for two minutes, 7 minutes, and after washing for three minutes.

tions, responses were reduced and excitability lowered. These observations suggest that a cholinergic system may be involved.

d-Tubocurarine. Tubocurarine (10^{-5}–10^{-4}) reduced and ultimately blocked the muscle responses. Both fast and slow components were sensitive to the curarization (fig. 2). Block was essentially the same whether stimuli were applied to the ganglion or to the preganglionic nerve fibers or to the isolated muscle. Tubocurarine acts on the Phascolosoma proboscis retractor much as on vertebrate striated muscle.

Atropine and adrenalin. Atropine (10^{-4}) reduced both responses, especially the fast one. Adrenalin (10^{-4}) showed no significant effects.

Veratrine. In dilute solution (2×10^{-6}–10^{-5}) the initial effect of veratrine sulphate was to make responses to single stimuli double or repetitive. This repetitive discharge was particularly noted for the fast wave and for the first response in a series. After some time (10–15 min.) both responses were reduced. At higher concentrations both responses were reduced directly. The observed effect of veratrine is more characteristic of its action on non-myelinated nerves than on muscle.

Acetylcholine. It has been shown previously that the proboscis retractors are sensitive to acetylcholine. When a drop of acetylcholine (5×10^{-5}–3×10^{-5}) in sea water was applied to the muscle locally there was a visible local contraction. When applied to the muscle at the proximal recording electrode, partial depolarization was observed with reduced superimposed action potentials (fig. 1 C). At higher concentrations ($10^{-4.5}$) block of conduction occurred.

Potassium. It was stated previously (Prosser et al., '51) that isotonic KCl blocks the fast but not the slow response. This statement was in error in that increasing the potassium in a drop of sea water at the proximal electrode to three or 4 times normal markedly reduced the responses, especially the fast wave. Twice normal potassium had little effect.

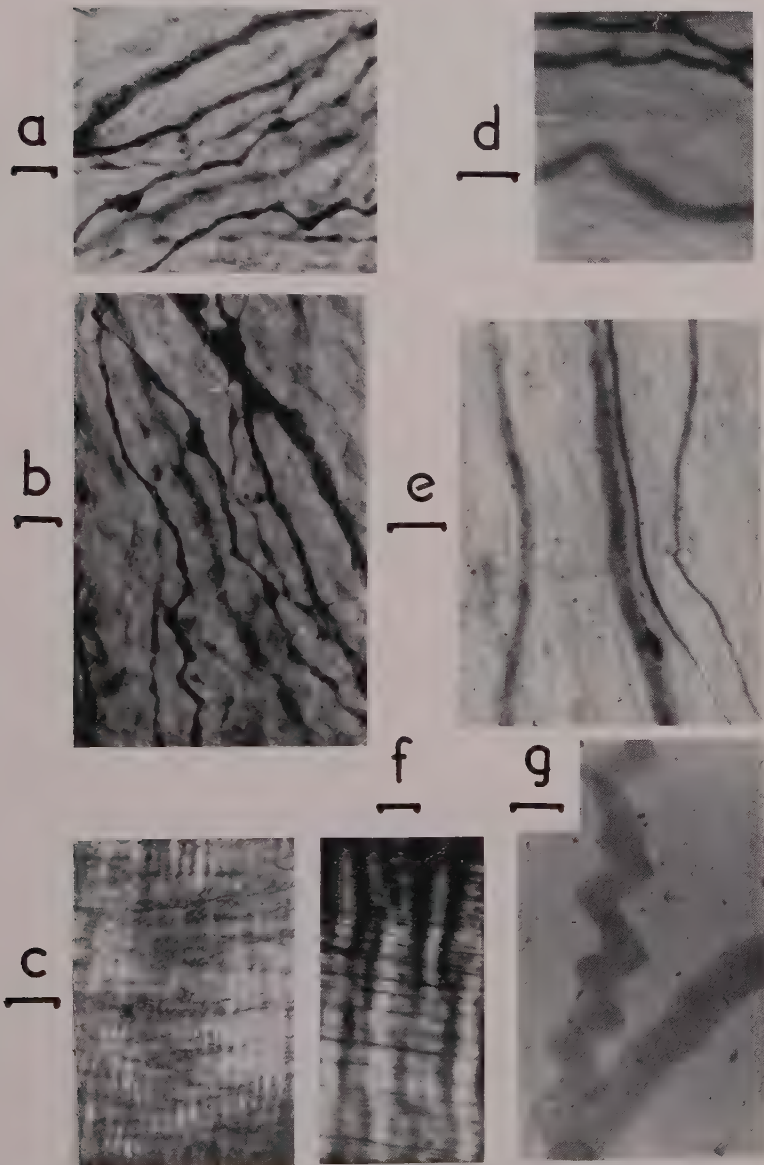

Fig. 3 Photomicrographs of Phascolosoma muscles, all photographed in living state except g. In a, b, c, d, e, f, nerves were stained with methylene blue; c, f photographed between crossed polaroids; g, photograph of portions of isolated contracted and relaxed muscle fibers. Magnifications indicated by brackets: a, b, f, 20 μ; c, 100 μ; d, e, g, 10 μ.

Excess potassium depolarizes and reduces the action potential much like acetylcholine.

Procaine. Procaine (10^{-3} but not 10^{-4}) applied locally reduced the action potential, the fast sooner than the slow, and ultimately blocked conduction.

The conclusion from the preceding observations (enhancement by eserine, block by procaine, block by tubocurarine, reduction of response by atropine, sensivity to acetylcholine, repetitive response after veratrine) is that conduction is probably in nerve elements and that the nerves for both fast and slow responses are cholinergic.

Histology. *Muscle fibers.* The structure of the muscle was observed in the living state, in sections and in macerations made in nitric acid and glycerol. The individual relaxed muscle fibers are 0.7 to 1.0 mm long and approximately 5 μ wide, although smaller fibers are sometimes found. The fibers taper to a sharp tip, sometimes forked. The muscle fibers are tightly bound together by a rich network of reticular connective tissue. When contracted the fibers fold and form zig-zag or spiral bands extending across the muscle. In this condition brilliant colored birefringence is seen when the muscle is examined between crossed polaroids (fig. 3 c, f). The macerated fibers may be straight or zig-zag according to whether they are fixed in a relaxed or contracted state (fig. 3 g). The spacing between folds is approximately 10 μ.

Nerve fibers. When methylene blue (0.1 ml of 0.1% solution in artificial sea water) was injected into the body cavity of active worms the muscles showed many spectacular nerve fibers in 12 to 24 hours. Similar but less complete staining was obtained by soaking the muscles in dilute solutions of methylene blue for several hours (fig. 3 a, b, d, e). The nerve fibers were readily distinguishable from the muscle fibers and ran in great abundance parallel to and between the muscle fibers and could be traced to their origin in the cerebral ganglion. Neurilemmal cells were present but no ganglion cells could be seen along the nerve fibers. The nerve fibers were in two size groups, larger ones approximately 2 μ

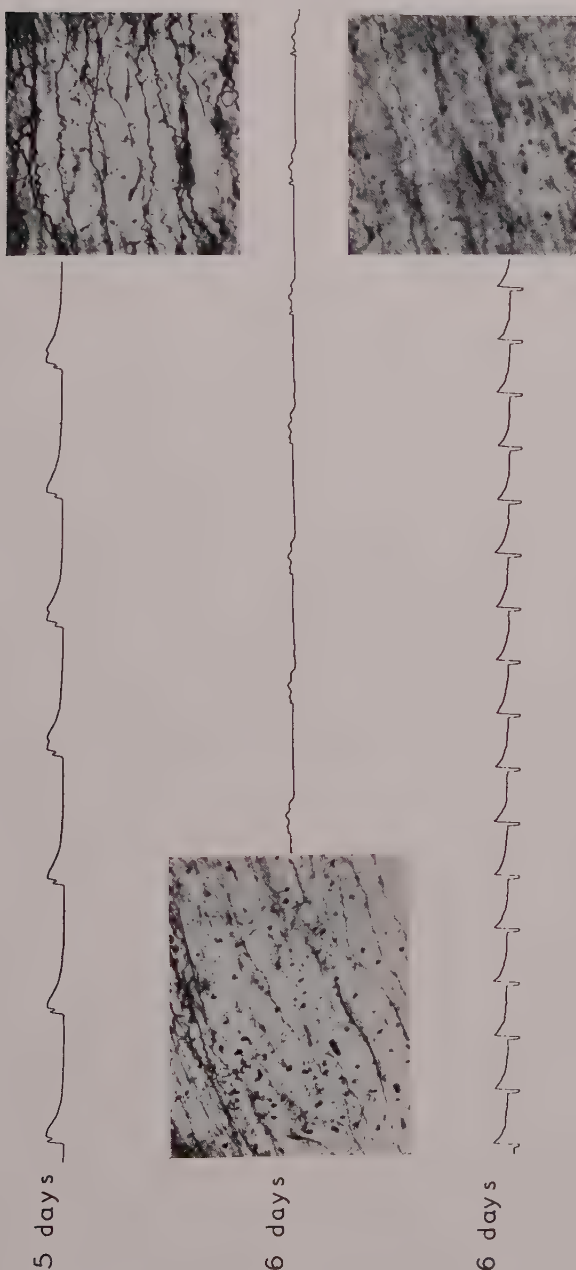

Fig. 4 Action potentials from degenerating muscles and photographs of same muscles after nerves were stained with methylene blue. Time in days after isolation from animal. No degeneration and no reduction in action potential seen in 5 days, partial degeneration and reduction in potential in first preparation at 6 days and complete nerve degeneration and reduction of response in second preparation at 6 days where only large stimulus artifact is recorded.

in diameter and smaller ones less than 1 μ in diameter (fig. 3 d, e). In serial photographs single nerve fibers could be traced for several millimeters without true branching and there is no doubt that the small fibers are separate entities and not branches from the large ones. Favorable preparations showed numerous little twigs which extended toward

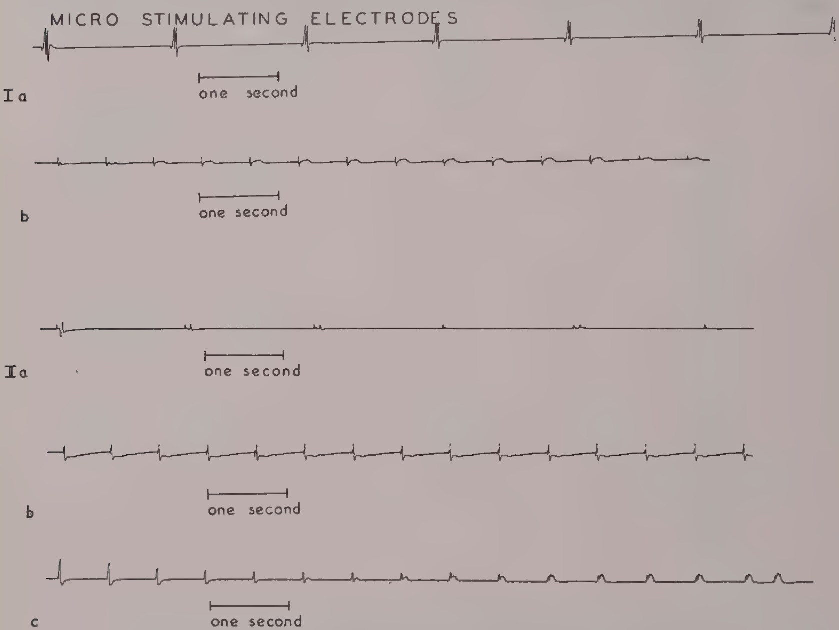

Fig. 5 Responses of two muscles with nerves stained and stimulated by 5 μ stimulating electrodes at different positions. I a, double fast response only; b, slow response only. II a, a fast response only; b, slow response only; c, fast and slow responses.

the muscle fibers but did not seem to penetrate. The nerve fibers were of similar size at both ends of the muscle, hence there can be virtually no attenuation.

Degeneration. It was previously reported that excised muscles left in the refrigerator for 5 to 7 days became unresponsive, the fast wave disappearing before the slow wave. We have recorded the action potentials and stained with methylene blue at different stages of degeneration. In gen-

eral, in 5 experiments involving 5 to 10 muscles each, the correlation between response and state of the nerve elements was excellent (fig. 4). When only slow responses were present, much degeneration of the nerve elements was noted, particularly of the large fibers. When no propagated responses remained, all of the nerve fibers were fragmented and granular. In such degenerated preparations the muscle fibers were in good condition and local contractions were readily obtained. In several series, degeneration in isolated muscles was compared with degeneration in muscles with the proboscis and ganglion left attached. Degeneration proceeded at about the same rate and thus it appears that the ganglion cells do not survive much better than do the nerve fibers when isolated from the animal.

Responses to stimulation by small electrodes. Muscles in which the nerves had been stained with methylene blue were excitable and showed both fast and slow action potentials. Such muscles (also unstained ones) were mounted under oil on a paraffin block where they could be observed with a dissecting microscope and the muscle was cut away to provide a narrow bridge containing relatively few nerve fibers. Pairs of very small (5 μ) steel electrodes, mounted 1 mm apart and carried on a micromanipulator, were brought near single fibers or groups of nerve fibers and the responses recorded with wicks on the intact muscle. Figure 5 shows that threshold shocks delivered by these very small electrodes could elicit in different positions either no response, both fast and slow responses, fast only or slow response only. The amplitudes of responses were very small and it is impossible to say with certainty whether or not only a large or a small nerve fiber was being stimulated at a given point, but discrete points were readily found at each of which only a fast or a slow response could be elicited. The fact that small responses were obtained when one or a few nerve fibers were stimulated indicates that the muscle is not a syncytial structure.

It appears, therefore, that the muscle is richly supplied with parallel nerve fibers, and that conduction fails when the

nerve fibers become degenerated. It is probable, from stimulation with small electrodes, that one type of nerve fiber elicits the fast and another type elicits the slow response. Whether the same muscle fiber can give both types of response we cannot say. The use of internal microelectrodes has thus

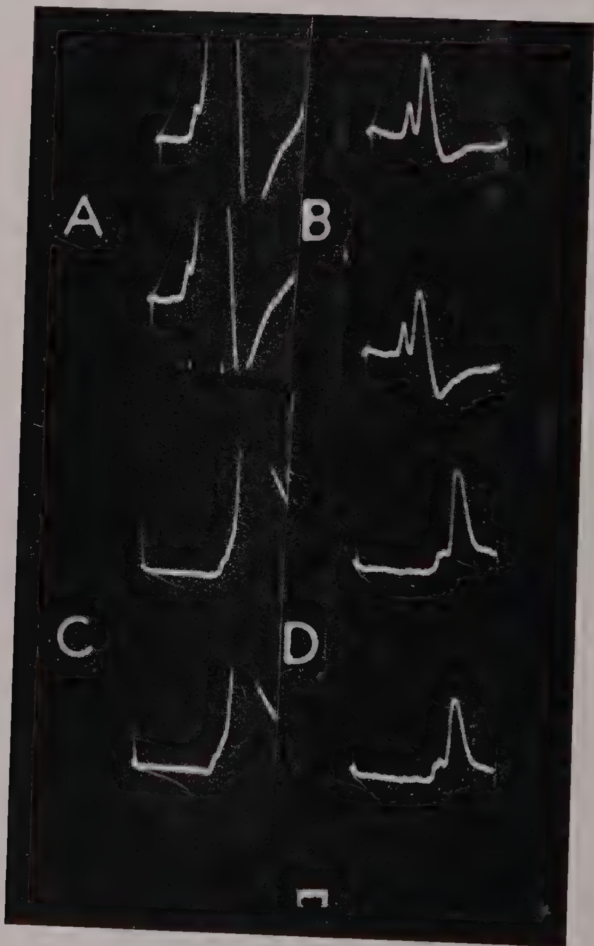

Fig. 6 Records with high-gain condenser-coupled amplifier. Two successive records at each of 4 electrode positions. Conduction distance for A and B 20 mm, for C and D 38 mm, showing propagation of "prespike." Separation of recording electrodes A 20 mm, B 16 mm, C 38 mm, D 12 mm, showing sharper "prespikes" with less separation. Time signal 0.01 sec.

far been rendered difficult by the small diameter of the fibers (5 μ).

High-gain recording. *"Prespikes."* Action potentials were also recorded with a high-gain condenser-coupled amplifier.

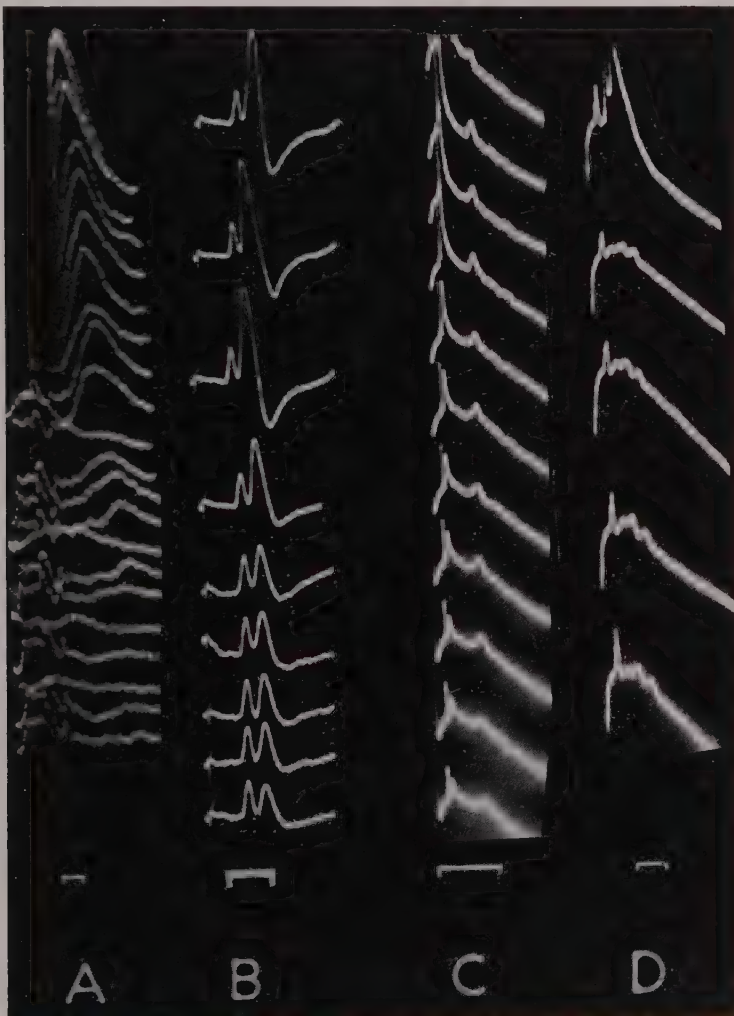

Fig. 7 Records of successive responses. A and B at fast sweep (time signal 0.01 sec.) to show fatigue of fast component of action potential and persistence of "prespike"; C and D at slower sweep (time signal 0.1 sec.) to show not only early but also late "prespikes."

At gains such that the fast and slow waves are incompletely recorded because they go off the oscilloscope face, there appeared consistently one or more "prespikes" ahead of the fast wave (fig. 6). These "prespikes" were best seen when the two recording wicks were not more than 5 mm apart; otherwise the "prespike" tended to be obscured by the fast wave of the muscle potential. On repetitive stimulation the

Fig. 8 "Prespikes" without muscle potentials in fatigued (A, B, C) or curarized (D, E) preparations. A at threshold for fast "prespike," B at higher intensity eliciting fast and slow "prespikes," C at higher gain and intensity showing compound nature of slow "prespike," time signal 60 cycle. D, conduction distance 35 mm, E conduction distance 21 mm, time signal 0.1 sec.; propagation of slow "prespike" shown.

fast wave fatigued rapidly leaving the "prespike" unchanged (fig. 7). The velocity of the "prespike" was the same as that of the fast muscle spike (about 1.5 m./sec. at 25°C.). Usually one "prespike" was evident but occasionally two or three were recorded. With slow sweep speeds another small amplitude potential was recorded just ahead of the slow muscle response. This second "prespike" was usually multiple, was seen best when the fast muscle response was reduced by fatigue, had a higher threshold than the fast

"prespike," and was conducted at the speed recorded for the slow wave (fig. 8). "Prespikes" for both fast and slow potentials are, therefore, recorded.

Effects of drugs. When d-tubocurarine (10^{-4}) was applied locally the muscle potentials were gradually eliminated but the "prespikes" persisted (fig. 9). Tetracaine (10^{-4}), applied in a drop of artificial sea water between stimulating and recording electrodes, rapidly reduced the muscle potentials, and also reversibly blocked the "prespikes" in 16 to 18 minutes (fig. 10). In tubocurarine block the "prespikes" remained whereas in tetracaine block both muscle potentials and "prespikes" disappeared.

It is concluded from the temporal sequence of the potentials, the velocities, the effects of fatigue, tubocurarine and tetracaine that the "prespikes" are impulses in nerve fibers which activate the muscle elements.

Interpretation of the muscle action potential[3]

On the basis of the preceding observations the proboscis retractors of Phascolosoma may be pictured as consisting

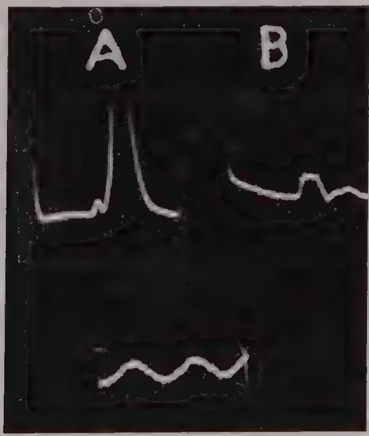

Fig. 9 Records of "prespikes" and fast muscle potential before (A) and after (B) 4 minutes local application of tubocurarine (10^{-4}), showing block of muscle potential only. Timer 60 cycle.

[3] We are grateful to Dr. Peter Stewart and Dr. H. J. Curtis for suggestions regarding the interpretation of the observed action potentials.

of many rows of overlapping parallel fibers, the muscle fibers of each row being activated successively by nerve impulses. Conduction through the muscle is, therefore, in the nerve fibers and no protoplasmic continuity between muscle fibers need be postulated. The presence of measurable action potentials in such a discontinuous system must be explained.

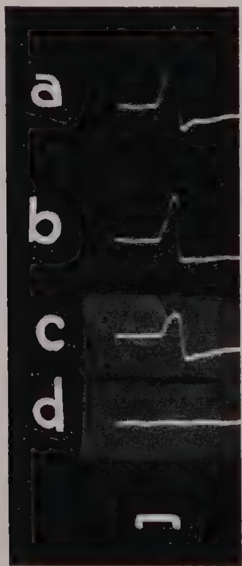

Fig. 10 Records of ''prespike'' and muscle potential; a, before treatment; b, 5 minutes; c, 7 minutes; d, 10 minutes after local application of tetracaine (10^{-4}); block of both ''prespike'' and muscle response shown. Time record 0.01 sec.

If each fiber were symmetrically and completely depolarized on activation, even though fibers were activated successively, no potential difference would be recorded between two external electrodes. However, if individual fibers were asymmetrically depolarized in series, each analogous to a continuous fiber, typical action potentials would be recorded. The active element or fiber must be long relative to its electrical space constant. If the resistance between cells is high the only low resistance path would be in the extracellular

fluid which serves as a long lead from an electrode to muscle fibers at some distance.

The first evidence favoring such an hypothesis is the geometric asymmetry. Individual fibers of this smooth muscle are approximately 200 times longer than wide, a greater ratio of length to width than in most striated muscle fibers. In extensive microscopic examination of the muscle, no indication of protoplasmic connection between fibers was obtained although fine reticular fibers binding muscle fibers together are abundant.

Conduction in the nerve fibers is sufficiently fast, relative to the duration of the muscle action potential that a number of muscle fiber doublets would add in series and the potentials must be recorded with external electrodes from hundreds of fibers in a three-dimensional structure. The shape and amplitude depend markedly upon the distance between recording electrodes. Increasing duration and amplitude, also change in shape of the action potential, with increased electrode separation are shown for both diphasic and monophasic recording in figure 11. The sign of diphasic action potentials reverses with direction of conduction; this can be interpreted as indication that each muscle fiber receives several nerve endings and activation can begin at either end.

Further evidence for short doublets was given by placing a muscle on a sheet of filter paper soaked with sea water and recording between this diffuse lead and a fine wire electrode which was moved away from the muscle at millimeter intervals. When proboscis retractors of Phascolosoma were used (7 series), the amplitude of the action potential diminished sharply as soon as the probe electrode moved off the muscle, and the amplitude fell to 10% of its initial value at 1 mm distance (fig. 12 Ia–d). When a long-fibered frog sartorius was used (7 series), the recorded action potential declined less in amplitude, was readily observed out to 7 mm away from the muscle and averaged 20% amplitude at 5 mm (fig. 12 IIa–d). These results indicate a much shorter doublet for the smooth muscle.

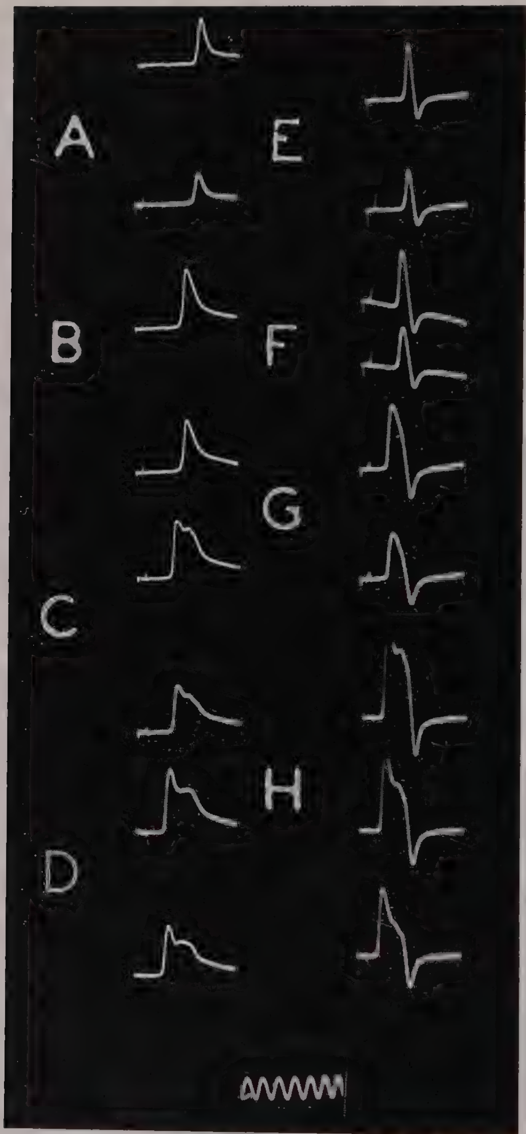

Fig. 11 Monophasic (A, B, C, D) and diphasic (E, F, G, H) low-gain records of muscle action potentials at increasing separation of recording electrodes. Two successive responses at each spacing of electrodes. Interelectrode distance A, 4 mm; B, 16 mm; C, 24 mm; D, 30 mm; E, 4 mm; F, 8 mm; G, 16 mm; H, 24 mm. Timer 60 cycle.

When records were made from a thin strip of frog sartorius in a saline field, triphasic responses, positive, negative and positive, were obtained as in a typical volume conductor

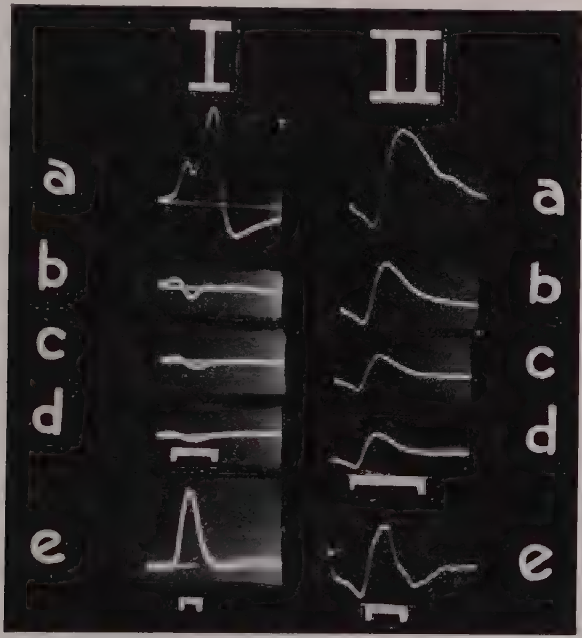

Fig. 12 Records obtained by a roving point electrode with reference to an infinite electrode (field of saline). Series I from Phascolosoma proboscis retractor, series II from frog sartorius. Ia point electrode on muscle, Ib, 1 mm; Ic, 2 mm; Id, 3 mm; from muscle. IIa on muscle, IIb, 1 mm; IIc, 5 mm; IId, 7 mm; from muscle. Ie record from another muscle, lower gain, point electrode on Phascolosoma muscle, typical monophasic response. IIe slower sweep than IIa–d, point electrode on frog muscle, triphasic response. Time records 0.01 sec.

(fig. 12 IIe). When the record was made from a Phascolosoma muscle of similar thickness there was no initial positivity and the response was nearly monophasic (fig. 12 Ie); as the point recording electrode was moved away, the record became diphasic (negative, then positive) quite unlike the frog muscle record (fig. 12). The two effects, (1) very rapid fall in amplitude with distance from the muscle and (2) the absence of triphasicity on the muscle with diphasicity off

the muscle, indicate the existence of many short batteries in the Phascolosoma muscle as opposed to the long-fibered frog muscle.

The presence of an injury potential might be cited as evidence for continuity. However between a crushed or KCl-treated end and an intact region resting potentials rarely exceeded 4.0 mv. (Prosser et al., '51). Similar low values are found for many other smooth muscles (Bozler, '41a, b). Under similar conditions a long-fibered frog sartorius gives injury potentials 5 to 7 times greater. Interpretation of such small resting potentials in such a complex electrical system is difficult. There are numerous liquid junctions and the long nerve fibers must contribute something to the injury potentials. We have observed similar values between a KCl-treated end and an intact region in strips of frog liver. The presence of injury potentials of the order of 3 to 4 mv. cannot be taken as proof of protoplasmic continuity.

DISCUSSION

The preceding evidence shows that the proboscis retractors of Phascolosoma are richly supplied with nerve fibers. Degeneration experiments, stimulation with small electrodes and the effects of drugs indicate that specific nerve fibers are responsible for conduction of signals eliciting the two components of the muscle action potential. High-gain amplification supports this interpretation since small prepotentials (nerve impulses) are observed; these persist on repetitive stimulation, are not abolished by tubocurarine but are blocked by tetracaine. Apparently the prepotential for the fast muscle spike arises in the large nerve fibers and the prepotential for the slow component of the muscle action potential in the smaller nerve fibers. The identity of response in both directions is readily explained by the lack of marked attenuation of the many parallel nerve fibers. It is concluded that conduction in the proboscis retractor muscle of Phascolosoma is in parallel nerve fibers, that one group of nerves elicit fast all-or-none muscle potentials whereas a second group

elicit graded endplate-type muscle potentials; both types of innervation appear to be cholinergic. The pattern is remarkably like that in vertebrate striated muscle. A diagram indicating an hypothetical scheme of innervation is given in figure 13.

The muscle action potentials are most readily explained on the basis of asymmetric depolarization of successive fibers.

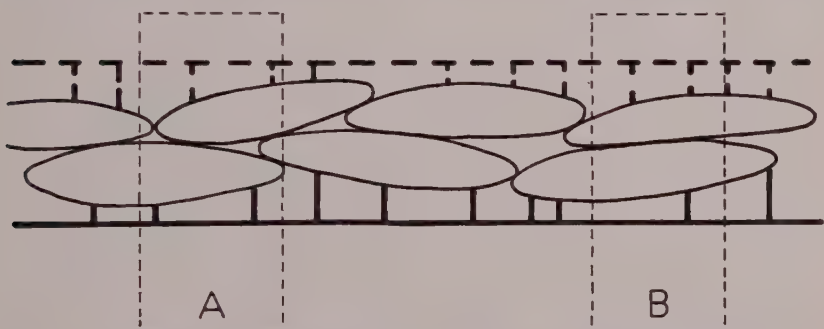

Fig. 13 Diagram of hypothetical scheme of conduction system in Phascolosoma proboscis retractor. Conduction from end to end by nerve fibers; muscle fibers indicated as discrete elements, each innervated at several points and capable of asymmetric depolarization. Two kinds of nerve fibers are indicated, for fast and slow responses; the two nerve fibers are diagrammed as innervating different muscle elements although there is yet no evidence indicating whether each muscle fiber receives one or both types of nerve fiber. Approximate dimensions: muscle fibers 0.7–1.0 mm long, 5 μ wide, nerve fibers 2 μ and 1 μ diameter. A and B represent recording electrodes.

This interpretation is supported by the large geometric asymmetry, the alteration in response with change in interelectrode distance, the rapid decline in response with reference to an infinite field, absence of triphasicity in volume conduction, and the small injury potential. The proposed interpretation of the monophasic action potential is essentially similar to that for records from the cerebral cortex (Bishop and O'Leary, '50). The Phascolosoma muscle is clearly different from Purkinje tissue of the heart where functional continuity from fiber to fiber is indicated (Curtis and Travis, '51; Draper and Weidmann, '51).

It would be of interest to know whether or not single muscle fibers receive multiple innervation and can give both types of action potential. One would also like to know the time and space constants of the muscle fibers. Answers to these questions await the use of internal microelectrodes. In any case, it is not necessary to postulate intercellular conduction or protoplasmic bridges. In these short-fibered, non-striated muscles conduction appears to be entirely nervous and when nerve fibers are eliminated only local muscle responses remain.

SUMMARY

The proboscis retractors of *Phascolosoma gouldii* are innervated from the cerebral ganglion by many parallel nerve fibers, some approximately 2 μ and some less than 1 μ in diameter.

When the nerve fibers are visibly degenerated, conduction is lost and only local contractions are seen.

Stimulation by small electrodes reveals local points which elicit only fast and others which elicit only the slow muscle potentials.

Records with high amplification reveal nerve impulses ahead of both fast and slow muscle potentials; the nerve impulses persist on fatigue of the muscle, they are blocked by tetracaine but not by tubocurarine.

The muscle response is blocked by tubocurarine, by procaine and tetracaine, and is sometimes made repetitive by veratrine. The muscle response, especially the facilitating slow component, is enhanced by physostigmine.

Individual muscle fibers are 0.7 to 1 mm long and 5 μ or less in diameter; they fold in a spiral or zig-zag on contraction.

The amplitude and form of the action potential change with interelectrode separation. The amplitude declines much more rapidly than in a long-fibered muscle as a point electrode is moved away from the muscle in a saline field and the sign of the response is not typical of a long volume conductor.

It is concluded that conduction in this smooth muscle is by parallel nerve fibers and that the muscle potential is best explained on the basis of asymmetric depolarization of successively activated elements.

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SOME COMPARATIVE BIOCHEMICAL ASPECTS OF CELL-FREE BACTERIAL LUMINESCENCE [1]

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THREE FIGURES

INTRODUCTION

Cell-free bacterial luminescence has recently been reported from this Laboratory. The dialyzed, particle-free extracts prepared from *Achromobacter fischeri* require $DPNH_2$ for light emission (Strehler, '53). Since then, two additional factors have been found to be required for a maximal luminescent reaction. One of these components is the flavin coenzyme, FMN[3] (Strehler and Cormier, '53; McElroy et al., '53), the other is a long-chain aldehyde (palmitic aldehyde) isolated from hog kidney cortex powders (Cormier and Strehler, '53; Strehler and Cormier, '54).

Since the described work was carried out with only one species of luminous bacteria, *A. fischeri*, it appeared to be of interest from the comparative biochemical point of view to determine whether the type of luminescent system described for this species is a general one or whether it is restricted to the one strain investigated. Because of the similar emission spectra and response to inhibitors among the various strains of luminous bacteria (van der Burg, '43; Spruit and van der

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[3] The following abbreviations are used in this paper: $DPNH_2$, reduced diphosphopyridine nucleotide; FMN, flavin mononucleotide; DPN, diphosphopyridine nucleotide.

Burg, '50; Johnson, Eyring and Williams, '42; see Harvey, '29, '52), one might expect to find similar biochemical requirements and mechanisms operative, although the mechanisms might equally well have been totally different, just as firefly and *Cypridina* luminescence differ from bacteria and from each other (Harvey, '17, '53; McElroy and Strehler, '49). In this communication are reported comparative studies of the luminescent systems obtained from 10 different strains of luminous bacteria. A number of factors have been examined in order to determine whether differences exist from strain to strain in the (1) extractability of the system, (2) requirement for diffusible factors, (3) Michaelis-Menten constants for the different intermediates, (4) activation energies for the different reactions, and (5) luminescence per unit weight of bacteria.

MATERIALS AND METHODS

Bacteria and culture methods. Ten different strains of bacteria were used in the present investigation. One of them, *A. fischeri,* was obtained from the American Type Culture Collection and the others were obtained through the generosity of Prof. C. B. van Niel. The identity of these 9 strains is not known but most of them appear to be different from one another as evidenced by the appearance of unstained preparations. Some strains appeared as long slender rods, some as very small cocci, while others were either coccobacilli or short plump rods (table 1 gives the appearance of each strain under the microscope). Furthermore, many of them differ in apparent intensity of their luminescence in liquid culture, in the amount of pigment secreted into the medium, and in the morphology of the colonies on solid media.

The medium used for the cultivation of all 10 strains in liquid cultures has been previously described (Strehler and Cormier, '53), and is used here with one modification (an addition of 5 mg of acid-hydrolyzed yeast nucleic acid per liter of culture medium). Stock cultures were prepared by allowing near maximum growth to be attained in liquid culture,

sealing 5-ml portions of this culture in glass tubes, and storing them at 0°C. (recommended by Dr. van Niel in private communication).

TABLE 1

Microscopic appearance and a comparison of the luminescence of ten strains of luminous bacteria

NO.	NAME [1]	DESCRIPTION	INTACT APPARENT LUMINESCENCE	EXTRACT LUMINESCENCE/MG DRY WT.
				(cts/5 sec.)
I	Bocor	Coccobacilli	***	372,000
II	1944	Short, plump rods (much larger than above strain)	*	1,410
III	Ayeng	Small cocci	***	342,000
IV	Kayser	Coccobacilli (larger than no. III)	***	187,000
V	Trucca	Coccobacilli	**	125,000
VI	1940	Large, plump rods (usually occurring in chains of 2–4)	*	83,500
VII	Bokm.	Small cocci	***	665,000
VIII	1950	Small cocci	*	2,040
IX	Gest	Coccobacilli	***	577,000
X	*A. fischeri*	Long, slender rods	***	274,000

[1] The first nine types were obtained from Dr. C. B. van Niel; the *A. fischeri* from American Type Culture Collection.

* Weakly luminescent
** Moderate intensity
*** Brightly luminescent
} visual observation during growth.

The wet, unstained bacteria were examined under oil. Luminescence/mg dry weight was determined on 2.0% extracts in the presence of saturating amounts of $DPNH_2$ (500 μg/ml), FMN (2 μg/ml), and palmital (18 μg/ml) in 0.1 M phosphate buffer, pH 7.0.

The 12-liter flasks which were used for the liquid culture of the organisms were aerated under aseptic conditions. When heavy innocula were used, maximum growth was obtained in 18–24 hours. The 9 strains received from Dr. van Niel were cultured at 15–16° C. *A. fischeri* was grown at room temperature (23°C.).

Preparation of active acetonized powders and water extracts of those powders has been described previously (Strehler and Cormier, '53), and is the procedure employed with all strains used in this investigation.

Light measurement. The light-measuring equipment (Strehler, '51) was a liquid nitrogen temperature photomultiplier (IP22) operated as a pulse-counting device. Routinely, 0.2–0.5 ml of bacterial extract plus 1.5 ml of 0.1 M NaH_2PO_4 (pH7.0) was used as a test system. Components of the luminescent system were added just prior to light measurement (total volume, 2.0–2.3 ml).

Reagents. $DPNH_2$ and FMN were obtained from Sigma Chemical Co. and heptaldehyde from Eastman Kodak Co. Octylaldehyde, pelargonaldehyde, and palmitic aldehyde were synthesized according to published procedures (Kobata, '35; Hurd and Meinert, '43; Rosenmund, '18).

EXPERIMENTAL RESULTS

Two per cent aqueous extracts (on a weight basis) of acetone dried powders obtained from the 10 strains were tested for their ability to luminesce before and after addition of known components of the luminescent system of *A. fischeri*. Fresh extracts will emit light for several hours after preparation, and after this time will luminesce only on addition of $DPNH_2$. In the presence of $DPNH_2$, FMN will increase the luminescence 5- to 23-fold depending on the strain used, while palmitic aldehyde will vary in its stimulating action 3- to 200-fold. Figure 1 illustrates the effect of adding increasing amounts of $DPNH_2$ to the extracts in the presence of excess FMN and palmitic aldehyde. For each concentration-versus-luminescence curve, increasing amounts of substrate were added to a single aliquot of the extract. Measurements were taken after each addition. This procedure did not significantly affect the results, since the volume of test solution was never increased by more than 10%. It was preferable because it was more economical of reagents and enzyme and because it was less subject to pipetting, optical, and other sources of cumula-

tive error. Figures 2 and 3 represent the effect of adding FMN and palmitic aldehyde, respectively, in the presence of an excess of the other two components. The data are presented in figures 1, 2, and 3 as Lineweaver-Burk ('34) type plots in order to make it convenient to determine the half-saturation value of each component and to compare the values

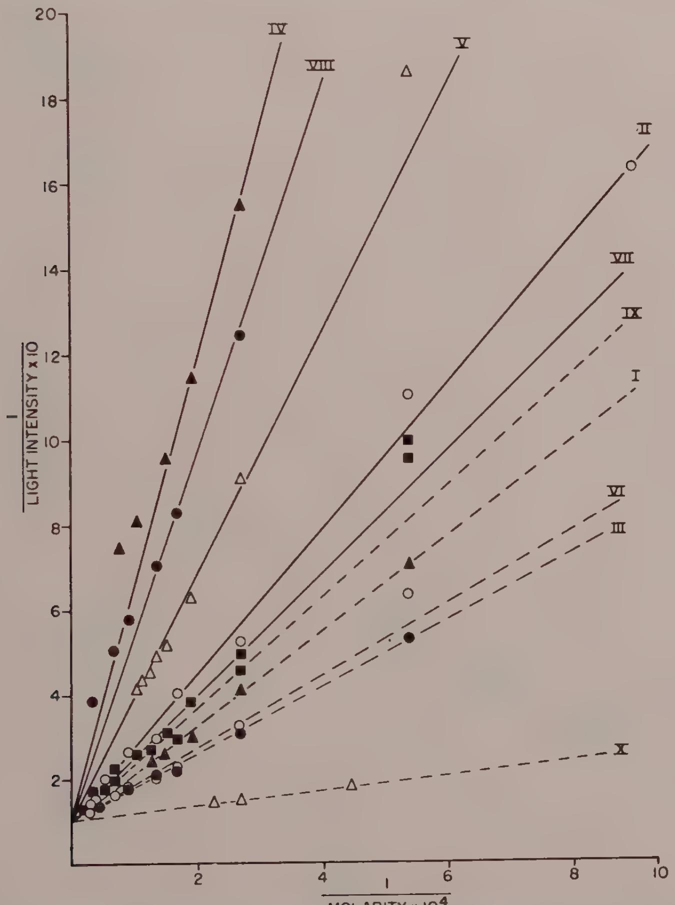

Fig. 1 Effect of $DPNH_2$ concentration on luminescence. To 0.3-ml portions of 2.0% aqueous extracts of the various luminous strains of bacteria was added 1.5 ml of 0.1 M phosphate buffer pH 7.0 + 4 μg of FMN + 36 μg of palmitic aldehyde. $DPNH_2$ was added as indicated, and the values for all strains were normalized to the same V_m for plotting.

obtained for the various strains used. In all three figures the plots are normalized so that the V_m (maximum velocity) is the same for each strain. In figures 2 and 3, each plot is corrected for zero concentration of FMN and palmitic aldehyde respectively — otherwise discontinuities occur in the

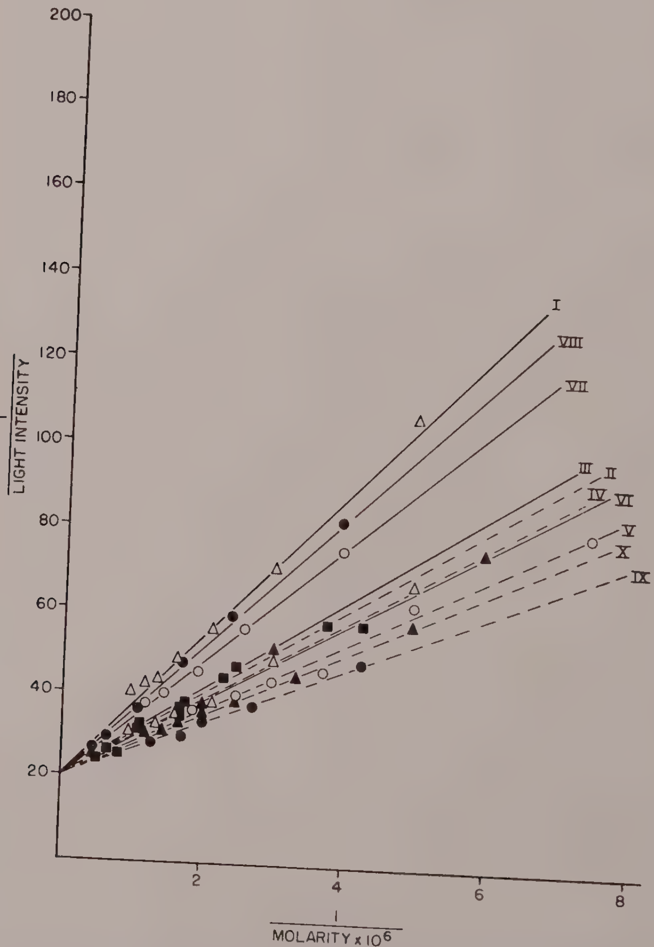

Fig. 2 Effect of FMN concentration on luminescence. To 0.3-ml portions of 2.0% aqueous extracts of the various luminous strains of bacteria was added 1.5 ml of 0.1 M phosphate buffer pH 7.0 + 750 μg of $DPNH_2$ + 36 μg of palmitic aldehyde. FMN was added as indicated and the value for all strains were normalized to the same V_m for plotting.

plots which correspond to initial low concentrations of these materials in the extracts.

A comparison of the slopes in figures 1, 2, and 3 indicates a variation in the Michaelis-Menten constant among different strains for $DPNH_2$, FMN, and palmitic aldehyde. Heptalde-

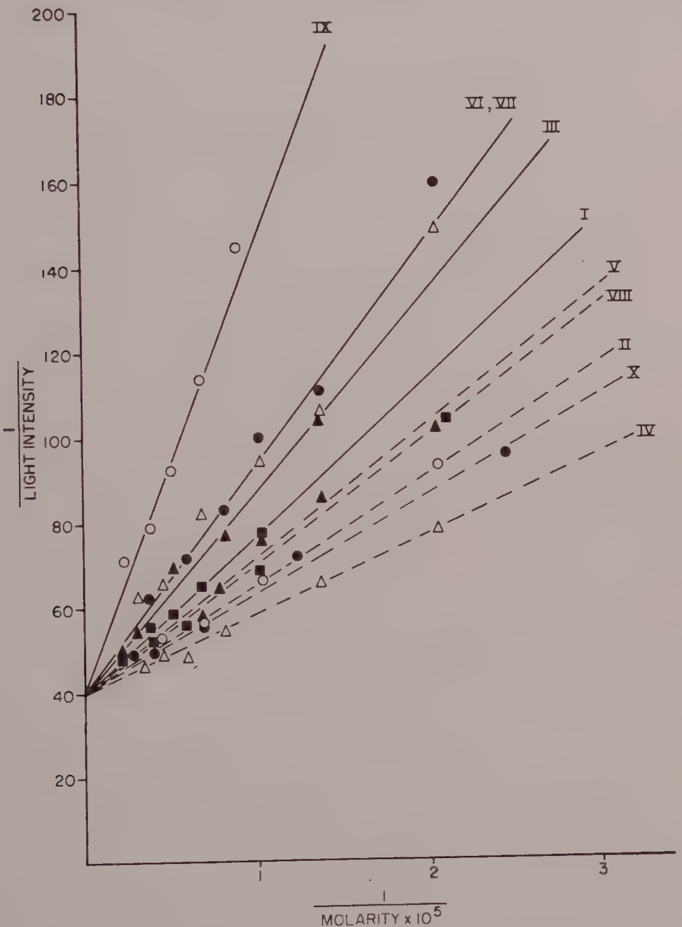

Fig. 3 Effect of palmitic aldehyde concentration on luminescence. To 0.3-ml portions of 2.0% aqueous extracts of the various luminous strains of bacteria was added 1.5 ml of 0.1 M phosphate buffer pH 7.0 + 750 μg of $DPNH_2$ + 4 μg of FMN. Palmitic aldehyde was added as indicated and the values for all strains were normalized to the same V_m for plotting.

hyde, octylaldehyde, and pelargonaldehyde were also tested for activity. All strains responded to each aldehyde tested although heptaldehyde was much less active for each strain than the higher homologues.

The apparent activation energy for the process when $DPNH_2$ is limiting (5–15% of saturation) was determined for each strain. $DPNH_2$ was made rate limiting in the presence of excess FMN and palmitic aldehyde. The test mixture was brought to the desired temperature and the luminescence immediately measured, points being taken at 2° intervals from 0° to 18°C. Since some inactivation of the system occurred due to depletion of the substrate or to higher temperatures (14°–18°C.), measurements were made at 10°C. before and after each determination at some other temperature. The average of two consecutive 10° readings was divided into the observed value and the quotient taken as the corrected value

TABLE 2

A comparison of the apparent activation energies, Michaelis-Menten dissociation constants, and temperature optima for $DPNH_2$, FMN, and palmitic aldehyde in ten strains of luminous bacteria

NO.	ΔH‡	$DPNH_2$ K_m	TEMP. OPTIMUM	ΔH‡	FMN K_m	TEMP. OPTIMUM	ΔH‡	PALMITAL K_m	TEMP. OPTIMUM
I	15,000	10.8	12	21,500	8.4	14	19,500	9.1	14
II	14,000	16.7	13	21,300	5.2	10	22,400	6.3	13
III	26,400	7.5	12	17,800	5.2	14	22,100	11.6	16
IV	22,400	51.0	12	18,100	4.7	14	20,500	4.75	16
V	22,500	30.0	13	19,300	4.0	14	20,000	7.9	16
VI	12,500	7.95	12	18,000	4.4	10	20,600	13.0	14
VII	18,600	15.0	12	20,700	7.0	14	22,500	13.0	14
VIII	12,700	37.5	12	16,000	7.5	12	17,700	8.0	16
IX	19,800	11.5	12	18,500	3.35	12	20,700	26.0	12
X	27,000	2.2	> 18	30,000	3.74	> 18	26,400	5.9	> 18

ΔH‡ Apparent activation energy.

K_m Michaelis-Menten dissociation constant $\times 10^5$ ($DPNH_2$), $\times 10^6$ (palmital), and $\times 10^7$ (FMN).

of the luminescence.[4] An Arrhenius plot was made from the values thus obtained in order to calculate the apparent activation energies and to make a comparison of the values obtained for the various strains. A straight line was observed when such a plot was made between 0° and 10°C. for 9 of the strains and between 0° and 16° for *A. fischeri.* A break occurred at these points in the curve which was probably due to partial inactivation of one or more of the enzymes at these higher temperatures. Values for the activation energies were calculated from the straight line portion of the curve.

Apparent activation energies for the reactions involving FMN and palmitic aldehyde were determined similarly to the $DPNH_2$ studies. The calculated $\Delta H^{\ddagger}$ values for $DPNH_2$, FMN, and palmitic aldehyde as well as the optimal temperatures for the reactions are presented in table 2.

The yield of luminescence per unit weight of dried bacteria in the different species was compared. A 0.4-ml portion of each bacterial extract was dried and weighed, and the maximum luminescence per milligram of dry weight was determined by adding saturating amounts of the three components required. The values obtained are presented in table 1.

DISCUSSION

From the data presented in this paper several conclusions are apparent. First, despite considerable variation in microscopic morphology and other physiological characters (such as colonial shape on solid media or pigment secretion), the biochemical requirements for luminescence of these various strains of luminous bacteria are, remarkably, identical. This, in all likelihood, indicates an identical mechanism for luminescence despite the small differences in emission spectra

[4] For obtaining the corrected values for luminescence at each temperature the following schedule of temperatures was used and equation (A) applied:

$$10°_1 \rightarrow X°_1 \rightarrow 10°_2 \rightarrow X°_2 \rightarrow 10°_3 \rightarrow X°_3 \rightarrow \cdot\ \cdot\ \cdot\ \cdot\ \cdot$$

where $10°_1$ = the first measurement at 10°C., $10°_2$ is the second, etc. and $X°_1$ = measurement at some other temperature.

$$\text{(A)}\ \frac{2X°_n}{10°_n + 10°_{n+1}} = \text{corrected relative value of luminescence.}$$

observed by earlier workers. Second, the appreciable differences in apparent activation energies for the same steps in different strains as well as the variation in the Michaelis-Menten dissociation constants among the various cultures suggest that the apoenzymes catalyzing the different reactions vary according to their biological source. Taking these two deductions from the observations together, it may be tentatively concluded that the biochemical steps, although identical, make use of catalysts with some range of properties.

With the exception of *A. fischeri,* the species here described were all isolated on the West Coast. The possibility that there may be some duplication in their genetic lines of origin is apparant. Their morphologies, on the other hand, probably indicate that they have arisen from somewhat diverse evolutionary lines.

Two alternative biochemical-evolutionary hypotheses can be set forth to account for the data being reported. First, the identical substrate requirements of the various strains may indicate a common ancestral line or, second, the ability to luminesce may have arisen repeatedly in genetically unrelated or only distantly related lines, or a combination of both. In general, we favor the second hypothesis for the following reasons:

1. All the intermediates thus far demonstrably necessary for luminescence of bacterial extracts, with the possible exception of long-chain aldehyde, are components not only of luminous bacteria but also of nearly all living things.

2. There does not now appear to be any special condition for the occurrence of luminescence except the terminal or light-emitting step; i.e., the demonstrable substrates (e.g., malate) and cofactors (DPN, FMN) appear conventional in their biochemical interactions in this system.

3. Since FMN may itself be the light-emitting molecule, no necessity exists for a series of mutations leading to a special "luciferin." Rather the production of a "luciferase" would seem to be the only obvious prerequisite to luminescence.

Horowitz ('45) has presented arguments that the likelihood of a complicated biochemical sequence evolving in the "forward" direction decreases as the number of steps increases. This tenet is well taken if the intermediate steps themselves have no survival value, but presumedly is not applicable if the partial step reactions per se confer some advantage on the organism. In the case of bioluminescent reactions among the bacteria, the steps presumably preceding the light emission step(s) are common to most living things. Thus the independent evolution of a catenary series of steps would not be prerequisite to light emission, and bioluminescence among the bacteria can be considered as a possibly fortuitous occurrence and recurrence with little or no necessary survival value (see Harvey, '40, '52, '53 for a discussion of luminescence in general).

Thus we would postulate that the ability to produce light in bacteria is the result of an accidental mutation in which the energy liberated by a terminal flavin autooxidase is channeled into the excited state of a molecule, perhaps flavin, which subsequently emits light. If in a natural environment this ability confers a selective advantage, this advantage may reside more in side biochemical effects than in the physical phenomenon of light emission.

SUMMARY

1. Ten different strains of luminous bacteria have been examined to ascertain biochemical differences and similarities among them.

2. All 10 strains were found to require $DPNH_2$, FMN, and palmitic aldehyde for optimal luminescence.

3. The Michaelis-Menten dissociation constants for these three factors were found to vary widely among the 10 strains examined.

4. Some variation among the activation energies for the different step reactions was found among the species.

5. Considerable variation was found among the strains with respect to the maximum luminescence obtainable per unit

dry weight and in the temperature optima of the luminescent system *in vitro*.

6. It is suggested that the ability to luminesce in bacteria has developed through mutations affecting one or a few enzymes mediating metabolic activities common to most, if not all, living things.

ACKNOWLEDGMENTS

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THE HEMOLYTIC AND ANTIHEMOLYTIC ACTION OF DODECYL AMMONIUM CHLORIDE [1]

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TWELVE FIGURES

It is now generally recognized that many hemolytic processes formerly considered to be non-osmotic in nature actually depend on an indirect osmotic mechanism which Wilbrandt ('41) has called "colloid osmotic." The first stage in this type of hemolysis is the induction by the lytic agent of a condition of free cation permeability in the erythrocyte; this is followed, in accordance with known principles of ionic exchange, by an increase in the internal osmotic pressure of the cell and by swelling of the latter to the point of hemolysis.

Among the many agents known to operate in this way are several anionic detergents; of these, sodium dodecyl sulfate has been most thoroughly studied by L. H. Love ('50). Many analogous cationic detergents are also known, and are likewise hemolytic (Fourneau and Page '14, Kuhn and Bielig '40, Bernheimer '47), but the mechanism of their lytic action has apparently not yet been investigated. One of them, dodecyl ammonium chloride (DACl) was accordingly chosen for the present study. This compound is hemolytic (Eggenberger, Harrimann, McClory, Noel, and Harwood '50), it is available

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in pure form, it has the same hydrocarbon chain length as sodium dodecyl sulfate, its general physical chemical properties are known (Corrin and Harkins '47, Debye '49), and an original method is available for its quantitative analysis even in very low subhemolytic concentrations. This analytical method forms the basis of a study to be reported elsewhere of the adsorption of dodecyl ammonium ions by human erythrocytes.

In the present paper it will be shown that in its essential features hemolysis by dodecyl ammonium chloride, like that by sodium dodecyl sulfate, is due to an induced cation premeability of the erythrocyte. In addition, particular attention will be given to two points not dealt with very adaquately in the existing literature on this type of hemolysis, namely the course of the prehemolytic swelling of cation-permeable cells, and the departures in certain respects of the behavior of cation-permeable cells from that commonly expected of them. The investigation of the first point required the development of a new method for distinguishing between the optical effects of swelling and hemolysis; that of the second point, a somewhat detailed study of the interaction of the hemolytic and antihemolytic activities of a single agent.

MATERIALS

Human erythrocytes, always from the same individual, were obtained by puncture of a fingertip. Crystalline dodecyl ammonium chloride of high purity was generously supplied by H. J. Harwood of Armour and Co., Research and Development Division.

METHOD

Because of the rapidity of the hemolytic processes with which this paper deals, and the relative slowness with which cells can be separated form their environment by centrifugation, direct measurement by chemical means of the time course of cation exchanges was not feasible. Photoelectric methods, on the other hand, have the required speed of response and

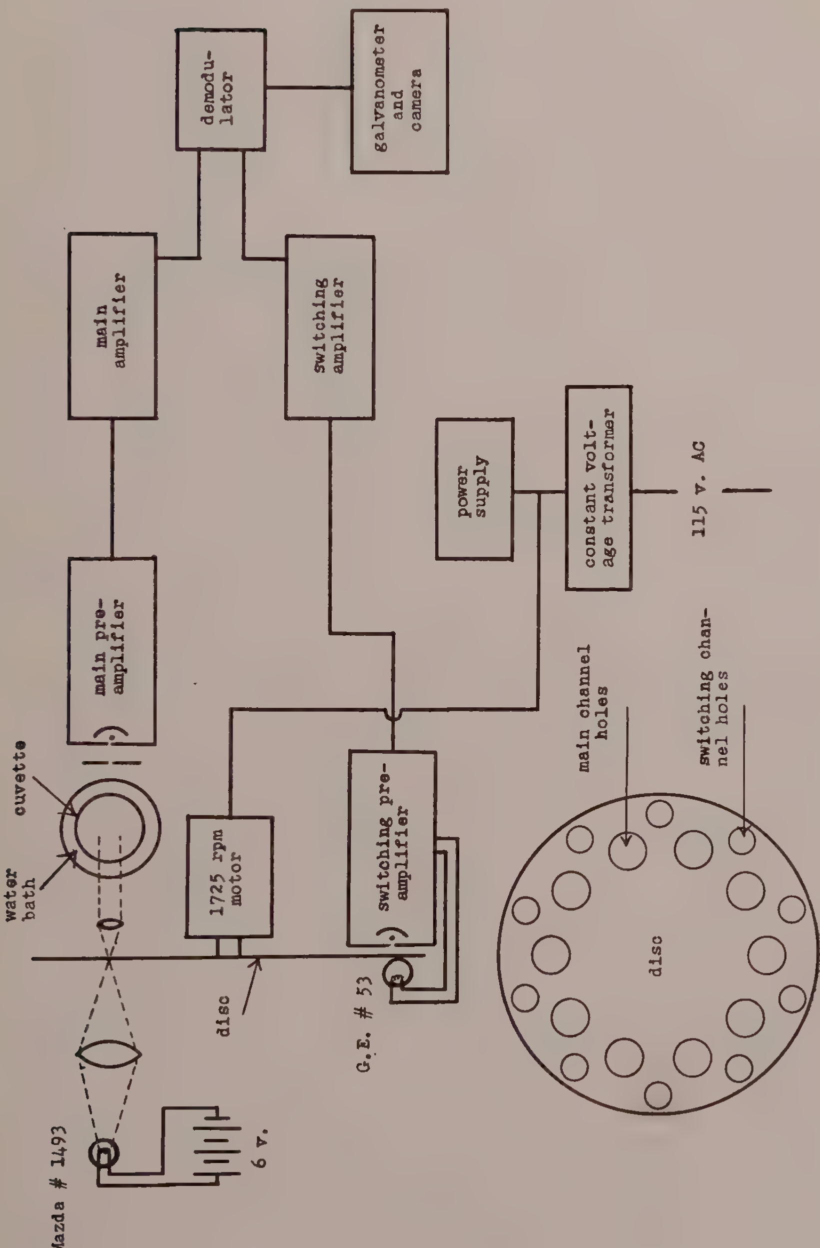

Fig. 1 Block diagram of the photometer.

were accordingly used. However, with the usual method of photoelectric observation of light transmission by suspensions of erythrocytes (Ørskov '35, Parpart '35), both hemolysis and swelling of intact cells cause light transmission to increase. For many purposes it is desirable to be able to separate these two processes and thus to know whether cells under given conditions are swelling or hemolyzing or both. The present method does this.

Exploratory measurements of the amount of light scattered by suspensions of intact erythrocytes under various conditions indicated that at suitable cell concentrations and angles of observation, the optical effects of swelling are opposite to those of hemolysis. However, the available photovoltaic cell and galvanometer were too insensitive for an extended study of hemolysis and therefore a more sensitive apparatus was built from a design of Dr. E. F. MacNichol Jr., to whom many thanks are due.

Figure 1 is a block diagram of the photometer. Light, interrupted 10 times per revolution of a sector disc, is rendered parallel by a lens and then passes through the cell suspension. The photocell in the main preamplifier is mounted so that it can be swung on the circumference of a circle around the sample as a center. A solid angle of observation of 16° is defined by a pair of apertures which limit the light falling on the photocathode. The light, flashing at 295 cps, causes an alternating current to flow through the phototube; this is amplified by conventional A C feedback technique. Another series of 10 holes in the sector disc interrupts a second beam of light, which in turn produces an alternating current through a second photocell. After A C amplification, the second signal activates a diode switch whereby the first A C signal is converted into a steady voltage, the size of which depends on the initial light intensity. A rapid mirror oscillograph is used to record the D C signal on moving photographic paper. The galvanometer deflections are linear with light intensity over the first 95% of the scale.

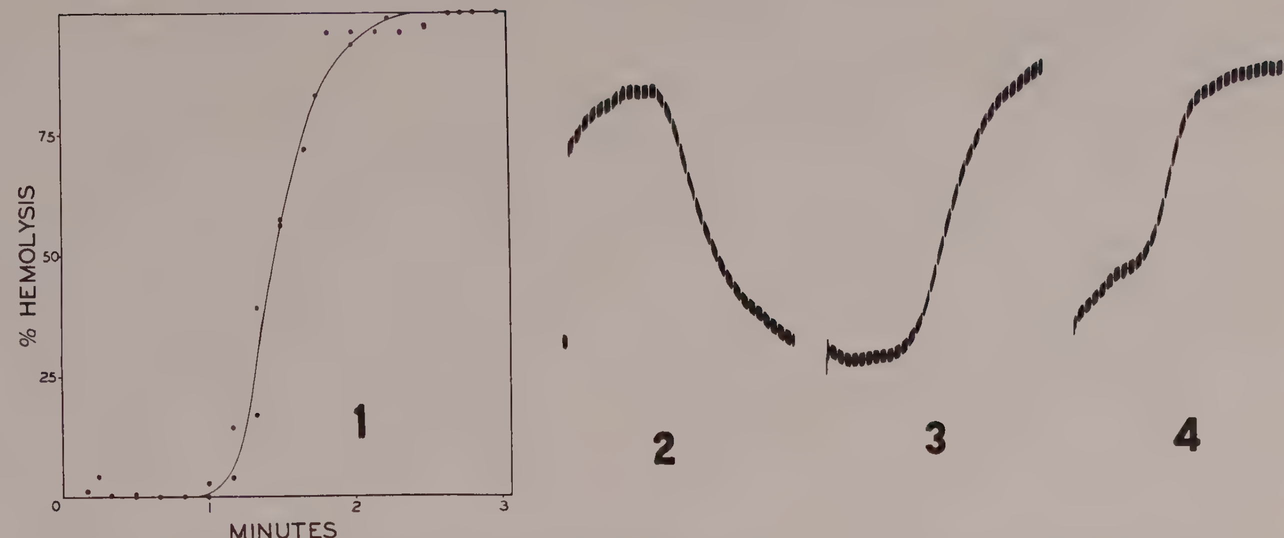

Fig. 2 Hemolysis in 0.3 M thiourea recorded in different ways (pH 7.4). (1) Hemolysis stopped at various times, and percentage hemolysis estimated colorimetrically. (2) Scattered light method, light increases are recorded upward; interruptions in the record occur at 6 second intervals. (3) Parallel light transmitted during thiourea hemolysis. (4) Diffuse light transmitted during thiourea hemolysis.

The photometer has two noteworthy features. First, it is sensitive only to light which has a 295 cycle component, and thus can be used in a moderately illuminated room. Second, the use of A C amplification avoids the problem of zero drift inherent in most D C amplifiers.

The utility of the scattered light method for separating osmotic swelling from hemolysis is demonstrated in figure 2, where the progress of thiourea hemolysis at pH 7.4 has been observed in 4 different ways. Curve 1, which shows the time-course of true hemolysis, was obtained by arresting lysis at various times by adding NaCl to erythrocytes suspended in 0.3 M thiourea. After centrifugation, the percentage of hemolysis was calculated from colorimetric measurements of the supernatant hemoglobin concentration, taking a distilled water lysate as 100%. Curve 2, obtained with the new apparatus, shows the changes in scattered light which result when the cell concentration is quite low and the photocell receives only light slightly deviated from the main parallel beam. Comparison of curve 2 with the standard curve shows that although hemolysis does not occur during the first minute the scattered light intensity rises during this time because of swelling of the cells, while after hemolysis begins, the light intensity falls. Thus under these conditions the changes in scattered light permit swelling to be separated from hemolysis; i.e., swelling causes an increase while hemolysis causes a decrease in scattered light.

Curves 3 and 4 illustrate another useful point. For curve 3, parallel light was passed through the curvette and changes in intensity were recorded, while curve 4 shows the effect of breaking up the parallel beam with a piece of ground glass before it entered the cell suspension. These records show that swelling appears as a rise with diffuse light while with parellel light the system is quite insensitive to swelling and changes due to hemolysis alone are recorded. These optical differences in the behavior of suspensions of hemolyzing cells are used in the following section.

Production of cation-permeable cells by DACl

Prior to about 1940 it was generally thought that the cationic composition of the interior of the erythrocyte remained different from that of the plasma by virtue of a physical impermeability of the cell to cations. Recent work with radioactive isotopes, however, has clearly shown that Na and K ions can normally pass across the erythrocyte surface, though at a slow rate commonly measured in hours or even days (Raker, Taylor, Weller and Hasting's '50, Sheppard and Martin '50, Solomon '52). Since the hemolytic processes reported here are measured in seconds, or at most in minutes, it is reasonable for present purposes to assume that during the times in question the normal erythrocyte is virtually cation-impermeable. If however, by artificial means, a free cation permeability should be induced, its addition to the free, passive, and rapid anion permeability known already to exist in the normal erythrocyte ought theoretically to lead to indefinite swelling and hemolysis of the cell in a physiological salt solution through the operation of simple osmotic laws and the Donnan membrane equilibrium (Jacobs and Stewart '47).

In order to decide whether a given hemolysin causes lysis by this mechanism of induced cation-permeability it has been found useful by Jacobs and his associates (Jacobs and Willis '47a, '47b, Love, Love and Jacobs '47, Willis, Love and Jacobs '47) to employ the following simple theoretical criteria by which the behavior of normal and cation-permeable cells are contrasted: (1) in a physiological salt solution the cation-permeable cell swells and hemolyzes, while the normal cell maintains its volume constant. (2) in solutions of non-penetrating non-electrolytes cation-permeable cells shrink to a much smaller volume than do normal ones. (3) in a suitable mixture of NaCl and a non-penetrating non-electrolyte, after the attainment of equilibrium, the addition of a small volume of saturated NaCl solution causes a rapid shrinkage and subsequent recovery of volume of cation-permeable erythrocytes;

but under identical conditions, normal cells do not recover their initial volumes. (4) on the addition of dilute alkali to a suspension of erythrocytes in mixtures of 0.3 M sucrose and 0.9% NaCl at pH 6.8, cation-permeable cells swell while normal ones shrink. (5) the permeability of cation-permeable cells to neutral molecules is usually increased to a marked degree above normal. (6) a concentration of a non-penetrating neutral substance in the surrounding medium so low as to have little effect on the hemolysis of normal cells by distilled

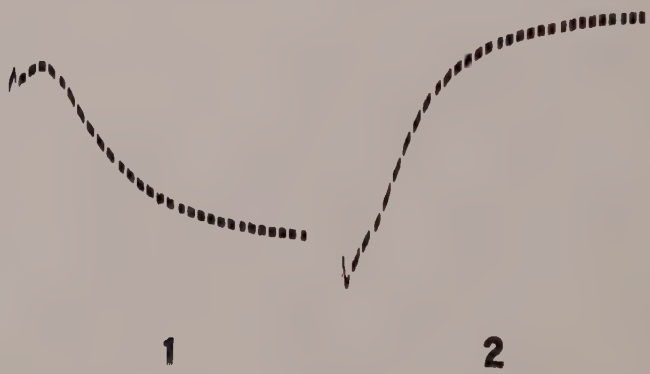

Fig. 3 Hemolysis in 0.9% NaCl due to the action of DACl. Interruptions in the records occur every second. (1) Changes in scatterd light during hemolysis. (2) Changes in parallel transmitted light during hemolysis.

water may completely prevent such hemolysis of cation-permeable cells.

One type of evidence suggesting that an induced permeability to cations is responsible for the hemolysis produced by DACl is the observation that erythrocytes treated with this agent always swell before they hemolyze. Curve 1 of figure 3 is a record of DACl hemolysis in 0.9%NaCl, obtained by the scattered light method. It will be noted that during the first few seconds the scattered light increases, i.e., the curve rises, thereby indicating a swelling of the cells before they lose their contents. Comparison of curve 1 figure 3 with curve 2 figure 2 shows, however, that the increase of the light scattered is not nearly as great as it is in the experiment with

thiourea. This is probably because the cell population is more heterogeneous with respect to resistance to the action of DACl, some of the cells undergoing hemolysis while others of the population are still swelling.

Support for this view is furnished by curve 2 figure 3 which was obtained under the same conditions of cell concentration, detergent concentration and pH as curve 1, but with recording changes in transmitted parallel light. Since in curve 2 the light intensity rises immediately after mixing, and since this arrangement of the optical system is peculiarly insensitive to

1 2 3 4

Fig. 4 Behavior of human erythrocytes in 0.3 M sucrose recorded with diffuse transmitted light. Ten second intervals. (1) Shrinkage of normal cells due to anion exchanges. (2) Excess shrinkage in the presence of DACl. (3) The effect of adding a small volume of saturated NaCl to a suspension identical with (2) after most of the shrinkage had occurred. (4) The addition of the same volume of saturated NaCl to a suspension identical with (1) after the attainment of equilibrium. The irregularities are due to the extreme flatness of these very shrunken but biconcave cells.

volume changes and records nearly pure hemolysis, this record indicates that some hemolysis occurred even during the first few seconds — hence the smallness of the rise in curve 1.

A second type of evidence for the suggested mechanism of DACl action is provided by the fact that in 0.3 M sucrose, human erythrocytes shrink to a very much smaller volume in the presence of DACl than they do in its absence. Figure 4 curve 1 shows the behavior of normal cells in sucrose and curve 2 shows the marked excess shrinkage in the presence of DACl, as recorded with diffuse transmitted light. This difference is in accord with the hypothesis of an induced

permeability to cations and a consequent loss of intracellular electrolyte to the suspending medium. The lesser degree of shrinkage of normal cells in sucrose is accounted for by the well known exchanges of anions without loss of cations (Jacobs and Stewart '47).

A third type of evidence appears in curve 3 of figure 4, which shows the rapid shrinkage and the subsequent return to equilibrium volume of the cells when a small amount of saturated NaCl solution is added to the suspension at the end of curve 2. The rapid decrease of volume, according to the theory, is due to the loss of water resulting from the increase in external osmotic pressure on the addition of the NaCl. The subsequent return to equilibrium volume is due to the entrance into the cells of some of the added NaCl which now behaves as a typical penetrating solute. The fact that the cells swell to slightly more than their volume before the addition of the NaCl is also in agreement with the theory, as this experiment was done at pH 7.4 where the equilibrium distribution of the diffusible ions is unequal (Jacobs and Stewart '47). Curve 4 of figure 4 is the control for curve 3 and shows the shrinkage without recovery of the original volume when NaCl is added to normal cells suspended in a sucrose solution. Irregularities appearing in the record in this case are due to the extreme flatness of such shrunken human erythrocytes.

A 4th type of evidence, namely, an increase in permeability to non-electrolytes such as is readily obtained with other agents which induce a condition of cation-permeability is also seen in the behavior of erythrocytes treated with DACl in solutions of non-electrolytes. The first curve in figure 5 is a record of the shrinkage of human erythrocytes in 0.3 M fructose in the presence of a hemolytic concentration of DACl. The shrinkage is what would be expected from the loss of intracellular electrolyte in the presence of a high degree of induced permeability to cations. Such cells hemolyze eventually when the experiment is sufficiently prolonged because of either a slowly developing, or a constant very low, permeability to fructose. Curves 2 and 3 were obtained under the

same conditions with pentaerythritol and erythritol respectively. The degree of permeability to cations is seen to be of the same order of magnitude in the three cases, in that the cells shrink at roughly comparable rates; the permeability to the non-electrolytes indicated by the later rises of the curves, however, is inversely related to the size of the molecules in question, as is to be expected in a series of lipoid insoluble substances such as this (Höber, '45). Curve 4 is discussed below.

It has been shown that DACl-treated erythrocytes meet 4 of the 6 criteria mentioned above. The protection against

Fig. 5 Behavior of human erythrocytes in solutions of different 0.3 M non-electrolytes but identical DACl concntrations. Ten second intervals. (1) Fructose. (2) Pentaerythritol. (3) Erythritol. (4) Glucose.

hemolysis in distilled water by low concentrations of sucrose, which is very clearly shown in the case of butyl alcohol hemlysis (Jacobs and Willis, '47a) has not been obtained with DACl because of complications due to the "protective" action of the latter substance itself; this will be discussed below. Attempts to demonstrate pure swelling on the addition of either dilute acid or alkali to erythrocytes suspended in DACl-NaCl-sucrose solutions at pH 6.8 were also unsucessful because complete hemolysis occurred so rapidly in all cases that a simple initial increase in volume could not be distinguished by the method employed.

The expected hemolytic effect of a pH change in the alkaline direction, caused by an altered Donnan distribution of ions is

probably masked by a much more potent factor, namely the increase of hemolytic ability of DACl with increasing pH. This is illustrated in figure 6 where the time for 75% hemolysis of a standard cell suspension in 0.9% NaCl with two different DACl concentrations is plotted as a function of pH. A shift of reaction toward alkalinity is thus considered as merely an increase of the ambient DACl concentration; this

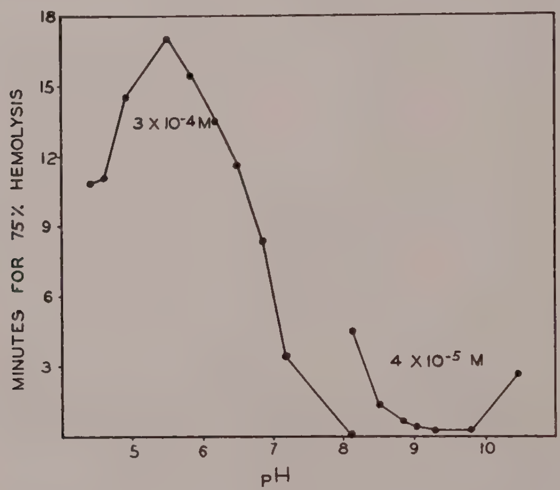

Fig. 6 The effect of pH on the hemolytic potency of DACl.

interpretation is reenforced by measurements of DACl binding as affected by pH (Love, '52) which are the subject of a later paper. The strong hemolysis which results on changing the pH in the acid direction is probably due in part to the theoretical Donnan effect, but it is also assisted by an entirely different mechanism, namely the removal of a characteristic protective effect of DACl which will now be discussed.

The antihemolytic action of DACl

Evidence already exists that certain hemolytic agents may also have an antihemolytic action (Jacobs, Willis and Stout

'49, Love '50). This action has been explained as being due to the tendency of such agents to form a layer at the surface of the erythrocyte which prevents or retards the escape of hemoglobin (Jacobs and Stout '50), or even perhaps the exchanges of ions (Love, '50) on which the hemolysis of cation-permeable cells depends. The removal of such a protective layer from sufficiently altered cells would automatically result in their prompt hemolysis.

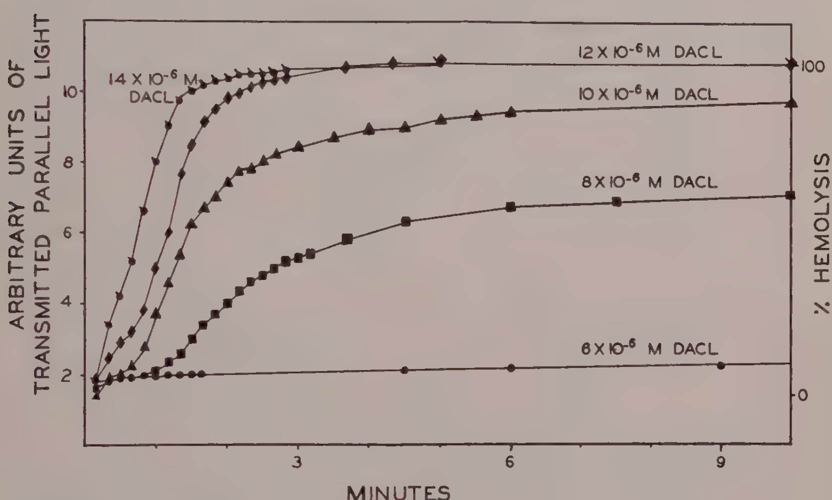

Fig. 7 Hemolysis by DACl in 0.9% NaCl at pH 8.0.

Such a factor appears to be involved in the results presented in figure 7 in which the intensity of parallel light transmitted by identical hemolyzing suspensions at various concentrations of DACl is plotted as a function of time. It will be noted that at intermediate concentrations of DACl the curves rise fairly rapidly, but flatten off short of complete hemolysis. In view of the "all or none" nature of hemolysis (Parpart, '31) and of the peculiarities of the optical system employed, it seems reasonable to assume that the initial rise in these curves represents complete hemolysis of varying fractions of

the cell population rather than partial hemolysis of all the cells. Those cells which remain appear to be protected in some way, and they are relatively stable under these conditions for more than 10 minutes, though they hemolyze eventually. The curves in figure 7 were obtained at pH 8, and other experiments at other pH values have demonstrated the generality of such a series, except that the concentration of DACl required to produce a given effect is a function of pH.

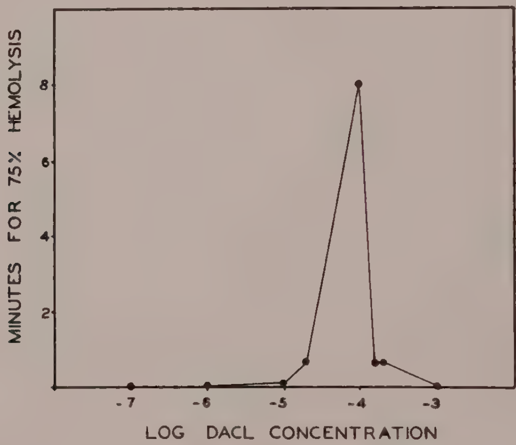

Fig. 8 The preservation of resuspended protected cells by the presence of DACl in the new medium.

The following experiment shows that DACl is itself the agent which prevents hemolysis in such cases. Erythrocytes were exposed for about 5 minutes to a slightly hemolytic concentration of DACl in 0.9% NaCl at pH 8. The protected cells remaining after the initial hemolysis were concentrated by centrifugation and aliquots of them were resuspended at pH 7.4 in 0.9% NaCl of different DACl concentrations. The time of 75% hemolysis was observed in each case. The times so obtained are plotted in figure 8 against the logarithms of the concentrations of the DACl in the new media. It will be noted that there is an optimum DACl concentration for the

preservation of such protected cells. Hemolysis is favored not only by higher concentrations of the hemolytic agent but also by lower ones, which, as will be shown in a later paper, tend to remove the bound DACl from the cell surface.

The protective effect of DACl may likewise be abolished by a change of pH alone, thus eliminating the time-consuming process of concentrating the cells by centrifugation. As has already been reported (Love, '52), the affinity of erythrocytes for DACl increases rapidly with increasing pH. Changes of pH therefore, are equivalent to decreasing or increasing the

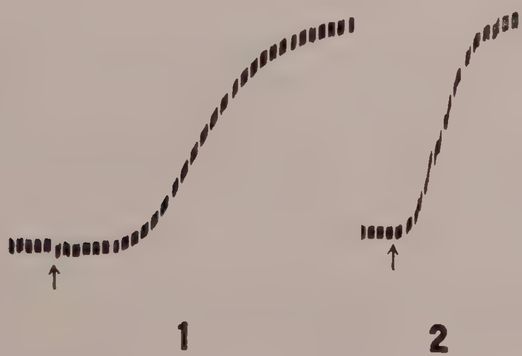

Fig. 9 Hemolysis produced by changing the pH in either direction, recorded with parallel light at one second intervals. (1) pH changed from 6.8 to 8.8. (2) pH changed from 8.8 to 6.8. The concentration of DACl for curve 1 was 7 times that for curve 2.

effective concentration of DACl in the medium. Changes in either direction can be lytic, but for different reasons. Figure 9 shows the effect of two such pH changes. Curve 1 is the hemolysis which results from adsorption of a lytic amount of DACl following a change of pH from 6.8 to 8.8. Curve 2 represents the hemolysis which results when a protective quantity of DACl is desorbed by a pH change from 8.8 to 6.8. The concentration of DACl in curve 1 was 7 times that in curve 2. It is because of the dominating effect of changes such as these that the theoretically simple relation between

pH and rate of hemolysis, well shown by erythrocytes made cation-permeable by butyl alcohol (Netsky and Jacobs, '39), fails to appear when DACl is the hemolytic agent. Similar complications, though with an opposite effect of pH on protection, have been reported for anionic agents (Love, '50, Jacobs and Stout, '50).

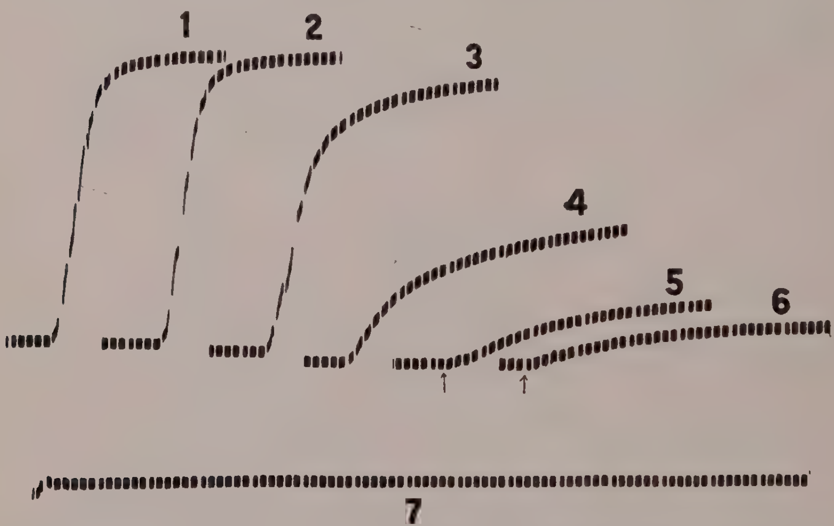

Fig. 10 The effect of the NaCl concentration of the hemolysis resulting from a pH change from 8.6 to 6.6. One second intervals. (1) 0.9% NaCl. (2) 1.8% NaCl. (3) 2.7% NaCl. (4) 3.6% NaCl. (5) 4.5% NaCl (6) 5.4% NaCl. (7) Control, 0.9% NaCl, pH not changed.

Having a simple method for the evaluation of the protective effect, namely, the exposure of cells to very slightly hemolytic solutions, followed by alteration of the pH toward acidity, the influence on the protective action of DACl of the NaCl concentration of the medium, and of the time of exposure have been studied.

Figure 10 summarizes the relation between NaCl concentration and protective effect. Cells were suspended at pH 8.6 in a subhemolytic solution of DACl in 0.9% NaCl to which variable amounts of extra NaCl had been added. After a constant time of exposure, the pH was changed to 6.6. It will

be seen that the rate and amount of hemolysis so obtained decrease with an increase in the NaCl concentration.

It might be supposed that the protective effect of the NaCl is here simply due to its osmotic pressure in the surrounding medium. That the situation is not so simple is shown by the fact that DACl is more hemolytic in sucrose solutions than in those of NaCl of the same osmotic pressure, despite the lesser degree of permeability of cation-permeable cells to

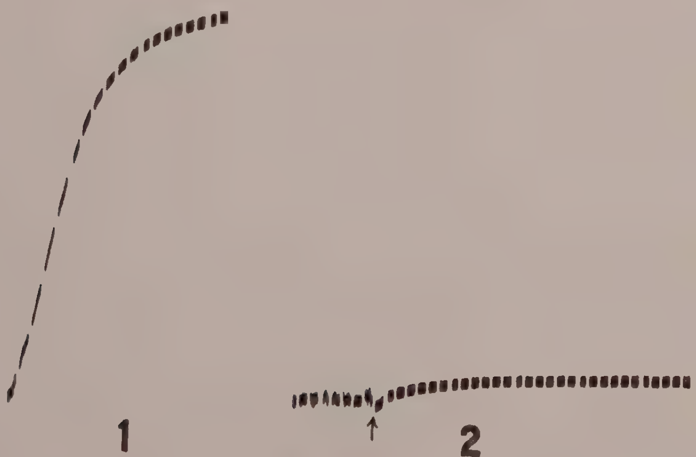

Fig. 11 The effect of a stepwise addition of DACl. One second intervals. (1) DACl added all at once, pH 8.6. (2) Two-thirds of the same volume of DACl solution added to an identical suspension. The last few seconds of a one-minute exposure are shown. The arrow indicates the addition of the remaining DACl solution which made the concentration equal to that in (1).

the former substance. The factor involved is probably the tendency of NaCl to cause micelle formation (Debye, '49), and thus to influence conditions at the cell surface in a manner that will be more fully discussed below. It may be noted that while the effect on hemolysis of added NaCl is complex and cannot be fully explained in terms of simple ionic equilibria, that of added sucrose in a medium of constant pH and constant concentration of NaCl, cells, and DACl, in which the protective effect is also constant, is in good agreement with predictions made by the usual theory of ionic and osmotic equilibria.

The time-courses of the protective effect and the hemolytic effect are probably different. Figure 11, curve 1 is a record of the hemolysis which results in certain conditions at pH 8.6 on the exposure of cells to DACl by the introduction of a small volume of a concentrated solution of the latter into the erythrocyte suspension. However, the introduction of two-thirds of the same small volume of concentrated DACl produces no marked effect. The beginning of curve 2 shows the amount of light transmitted one minute after such an addition. At the arrow the remaining one-third of the solution was now added, making the concentration of DACl exactly the same

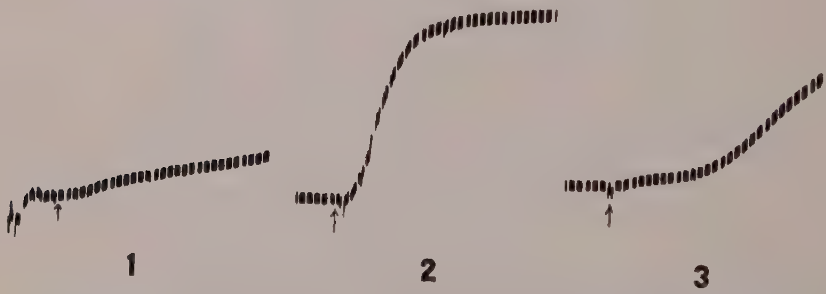

Fig. 12 The effect of time exposure to DACl on the hemolysis resulting from a pH change from 8.8 to 6.8. One second intervals. (1) Exposed for 5 seconds. (2) Exposed for 80 seconds. (3) Exposed for 320 seconds.

as that of curve 1. It will be noted that under these conditions of a preliminary exposure of the cells to a subhemolytic concentration of DACl, they are protected against hemolysis by an otherwise hemolytic solution.

The time-course of the development of the protective effect was also studied in another way. Cells were exposed to a subhemolytic DACl solution at pH 8.6, and after a variable time of exposure the pH was changed to 6.7. During the first 80 seconds the cells were found to become increasingly sensitive to the pH change but after that time they became increasingly resistant to it. Figure 12 shows typical results of such an experiment. Curve 1 was obtained after 5 seconds of exposure, curve 2 after 80 seconds, and curve 3 after 320

seconds. This experiment is interpreted as indicating that the lytic process takes an appreciable but short time to develop, whereas the protective effect reaches its maximum more slowly. This interpretation will explain the records in figure 11 where DACl was added either all at once or in two steps spaced a minute apart.

If the time-courses of the protective and hemolytic effects are different, it is reasonable to assume that the fundamental processes underlying them are also different. L. H. Love ('50) postulated such a dissimilarity as the explanation of the very peculiar influence of temperature on hemolysis by sodium dodecyl sulfate.

The effect of NaCl concentration on the protective action of DACl recalls the well known fact that increasing the ionic strength in a given solution of an ionic surface-active agent promotes the formation of micelles (Corrin and Harkins, '47, Debye, '49). Although in the work here reported the critical concentration for micelle formation in the body of the solution was at no time exceeded, it is entirely reasonable to suppose that something akin to micelle formation may have occurred at the cell surface; that this occurrence was facilitated by an increase in the salt content of the suspending fluid; and that in its presence DACl could not have a simple hemolytic effect.

In studying DACl hemolysis in solutions of different non-penetrating non-electrolytes it was found that these substances could be divided into two groups according to the type of hemolysis curves obtained. One group comprises maltose, fructose, pentaerythritol, and erythritol; sample curves involving three of these substances have already been presented in figure 5. The other group consists of sucrose, glucose, mannitol, and inositol; hemolysis curves obtained with them under exactly the same conditions are of a very different nature. A typical one for glucose is curve 4, figure 5, which was obtained under the same conditions as the first three curves in the same figure. In general, as the concentration of DACl in solutions of members of the first group of

substances is increased, the whole process of shrinkage, swelling and hemolysis is simply accelerated. With the second group, at low concentrations of DACl, the cells behave normally, i.e., they first shrink to a very small volume through the loss of intracellular electrolyte, and eventually swell and hemolyze as they become permeable to the external nonelectrolytes. But as the DACl concentration is increased, the pattern of hemolysis changes and an initial very rapid partial hemolysis is followed by slow hemolysis of the remaining cells. This relation is shown in figure 5, curve 4. Furthermore it has been found that increasing fractions of the cell population are involved in the immediate partial hemolysis as the DACl concentration is increased. No explanation of these differences is offered here except that in the case of substances of the second group the cells seem to be protected from the hemolytic action of DACl very early in the hemolytic process, while in the case of those of the first group, the cells behave in an uncomplicated manner. In both types of nonelectrolyte solutions DACl is more lytic than it is in NaCl solutions, which strongly favor micelle formation.

Whether the removal of a protective agent from protected cells merely uncovers an injury previously produced during adsorption, or whether the injury is caused by the actual removal has not been decided. The exact relation of pH to the protective effect of DACl has also not yet been determined, though it has already been mentioned that the protective effect, when present, can be abolished by changes of pH toward acidity. It is of interest in this connection to note that two anionic agents, sodium dodecyl sulfate (Love, '50) and sodium oleate (Jacobs and Stout, '50) have been reported to show their greatest protective action at acid reactions, and abolition of protection by increased alkalinity.

ACKNOWLEDGMENT

It is a pleasure to acknowledge my indebtedness to Prof. M. H. Jacobs for his encouragement and helpful criticism

during this work. I am also indebted to the Public Health Service for a predoctoral research fellowship.

SUMMARY

1. The action of a typical cationic detergent, dodecyl ammonium chloride, (DACl), on human erythrocytes has been investigated by studies of the type of hemolysis produced by it. DACl appears to be hemolytic primarily because it makes the erythrocyte freely permeable to cations.

2. With a newly developed method for the separation of the optical effects of swelling and hemolysis, it has been shown that human erythrocytes in a physiological salt solution always swell prior to hemolysis by DACl.

3. Hemolytic concentrations of DACl cause erythrocytes in an isosmotic sucrose solution to shrink strongly before they swell and eventually hemolyze.

4. On the addition of a small amount of NaCl to a suspension of DACl-treated cells in a suitable sucrose-NaCl solution, they first shrink almost instantly and then recover their volumes in times measured in seconds; i.e., NaCl behaves osmotically as a typical rapidly penetrating substance.

5. DACl increases to a remarkable degree the permeability of erythrocytes to normally non-penetrating non-electrolytes.

6. Under suitable conditions DACl protects erythrocytes from its own hemolytic action. Desorption of the agent from protected cells by dilution of the medium with 0.9% NaCl or by changing the pH toward acidity causes immediate and rapid hemolysis.

7. Within certain limits increasing either the ionic strength of the medium or the time of exposure to DACl favors protection. It is suggested that the protective action of DACl may be due to something akin to micelle formation at the surface of the cell which prevents the escape of hemoglobin, or reduces the degree of induced permeability to cations or of normal permeability to anions.

8. The action of DACl on erythrocytes in solutions of 0.3 M maltose, fructose, pentaerythritol, or erythritol is different from that on similar cells in solutions of sucrose, glucose, mannitol, or inositol, in that the latter substances favor protection very early in the hemolytic process.

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COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

TRANSPORT OF PHENOLSULFONPHTHALEIN DYES IN ISOLATED TUBULES OF THE FLOUNDER AND IN KIDNEY SLICES OF THE DOGFISH. COMPETITIVE PHENOMENA[1]

ROY P. FORSTER, IVAR SPERBER[2] AND JOHN V. TAGGART

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Previous studies in the chicken (Sperber, '53) have shown that the various phenolsulfonphthalein dyes are excreted by the renal tubules at widely differing rates. Phenol red is transported at the highest rate and, in descending order, are the following: xylenole blue, chlorphenol red, bromchlorphenol blue, bromcresol purple, bromphenol blue and bromcresol green. The various dyes are capable of competing with one another for the excretory transport mechanism. In general, those which are excreted at the highest rates are least

[1] This work was supported by grants from the Rockefeller Foundation.

[2] Ulric Dahlgren Memorial Fellow.

effective as competitive inhibitors, while those excreted at the lowest rates are the most effective competitors. These observations have now been confirmed and extended in *in vitro* kidney preparations of the marine teleost (winter flounder, *Pseudopleuronectes americanus*) and in the elasmobranch (dogfish, *Squalus acanthias*). In the former, the accumulation of dye in the lumina of isolated tubules was visualized directly. In the latter species, the effects of two of the dyes on the accumulation of p-aminohippurate were studied using kidney slices.

Transport in isolated tubules

The isolated tubule technic was as previously described (Forster, '49; Forster and Taggart, '50). Phenol red accumulated rapidly, giving a yellowish to red color in the tubular lumen. Chlorphenol red yielded an intense bluish-red color, while the accumulated xylenole blue was distinctly yellow. Bromcresol purple resulted in a faint purplish color. Bromchlorphenol blue and bromophenol blue could be detected in the tubular lumina only after an hour or more. Bromcresol green rarely accumulated in detectable amounts.

TABLE 1

Influence of bromcresol green (BCG) on chlorphenol red (CPR) accumulation in isolated flounder tubules

CPR (mg %)	0.6	0.6	0.6	0.6	0.6
BCG (mg %)	—	0.04	0.1	0.2	0.4
Time (min.)	Activity of CPR accumulation				
15	+	±	±	0	0
30	+++	++	+	±	0
60	++++	++++	++	++	±

The accumulation of chlorphenol red was graded at the indicated times as follows: 0 no accumulation, ± doubtful accumulation, + definitely detectable concentration, ++++ maximal accumulation.

The effect of bromcresol green on the transport of chlorphenol red is shown in table 1. Bromcresol green definitely inhibits chlorphenol red accumulation when the molar concentrations of the dyes are respectively 1 : 9. A similar inhibition of phenol red transport by bromcresol green was observed. The inhibitory effect of bromcresol purple was found to be distinctly less than that of bromcresol green.

Studies in dogfish kidney slices

p-Aminohippurate (PAH) and the phenolsulfonphthalein dyes appear to be excreted by the same tubular transport mechanism (Smith,

'51). Consequently, two of the dyes were examined for their effects on the accumulation of PAH in kidney slices of the dogfish. The slice technic was as previously described (Cross and Taggart, '50), except that an "elasmobranch saline" (Richards, '36) replaced the usual suspending medium. As is shown in table 2, bromcresol green com-

TABLE 2

Inhibition of PAH accumulation in dogfish kidney slices by bromcresol green (BCG) and phenol red (PR)

EXPER. NO.	RELATIVE CONC. IN MEDIUM × 10^4 M		RESPIRATION qO_2	PAH ACCUM. S/M
	PAH	*BCG*		
1	0.6	..	0.80	11.9
	0.6	2.4	0.75	0.8
	0.6	1.2	0.82	0.8
	0.6	0.6	0.76	0.8
	0.6	0.3	0.77	1.0
	0.6	0.15	0.80	3.8
	PAH	*PR*		
2	0.6	..	0.72	10.7
	0.6	2.4	0.71	5.1
	0.6	1.2	0.68	6.6
	0.6	0.6	0.76	6.6
	0.6	0.3	0.67	7.0
	0.6	0.15	0.69	7.5

Slices of dogfish kidney were incubated in Warburg vessels in media containing the indicated relative concentrations of PAH and dye. Temperature 25°C.; oxygen in the gas space; time 40 minutes. The qO_2 indicates cumm of O_2 consumed per milligram of slices per hour. PAH accumulation is expressed as the concentration of PAH in slices/medium (S/M). In estimating PAH in filtrates of the slices and media, appropriate corrections were made for light absorption by the dye.

pletely inhibited PAH accumulation at a molar concentration ratio of 0.5:1. In contrast, phenol red at a molar concentration ratio of 4:1 depressed the accumulation of PAH by only 52%. Neither dye had a significant effect on cellular respiration.

DISCUSSION AND SUMMARY

It is of interest that the relative rates at which the various phenolsulfonphthalein dyes are transported in the isolated tubule of the flounder parallel those previously noted in the intact kidney of the chicken. Furthermore, bromcresol green, the dye within this series which is least efficiently transported, has proved to be a potent competitive inhibitor of both chlorphenol and PAH transport.

In the course of the studies with the isolated tubules, it was noted that the use of chlorphenol red resulted in more uniform preparations than were obtained with phenol red. The bluish-red color of the former is more readily perceived and the stability of the color within the range of pH encountered in these preparations is a distinct advantage.

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INFLUENCE OF ANOXIA UPON HEMATOPOIETIC CELLS OF TADPOLES EXPOSED TO X-IRRADIATION OR COLCHICINE [1]

BENNET M. ALLEN, OLE ARNE SCHJEIDE AND RONALD PICCIRILLO
Atomic Energy Project, School of Medicine, University of California at Los Angeles

ONE FIGURE

INTRODUCTION

It has been shown (Allen, Schjeide and Hochwald, '50, '51; Schjeide and Allen, '51) that damage resulting from 500 r of x-irradiation given to the hematopoietic tissues of tadpoles of *Rana catesbiana* occurs mainly in those cells undergoing mitosis. When the cells reach the point at which they would normally divide, they

[1] This article is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

break down. It has been further shown that the rate of mitosis is strongly influenced by temperature (Allen, Schjeide and Hochwald, '50, '51), and that as a consequence, low post-irradiation temperatures retard the appearance of this damage.

In this paper we show that anoxia also suppresses cell division and thereby retards x-irradiation damage; the period of suppression occurring in rough proportion to the degree of anoxia.

MATERIALS AND METHODS

A total of 204 tadpoles of *Rana catesbiana* (80–90 mm in length) were divided into two groups which were subgrouped and treated as follows:

Group I: Given 500 r of x-irradiation.

- (a) Irradiation only, no period of anoxia.
- (b) Irradiation followed immediately by one hour of anoxia and with a second hour of anoxia 12 hours later.
- (c) Irradiation followed immediately by one hour of anoxia and one hour of anoxia at 6, 12, and 18 hours after irradiation.
- (d) Anoxia for one hour during which time the irradiation was administered. A second hour of anoxia administered at the end of 12 hours.
- (e) Anoxia for one hour during which time irradiation was administered. Additional one hour periods of anoxia at 6, 12, and 18 hours post-irradiation.

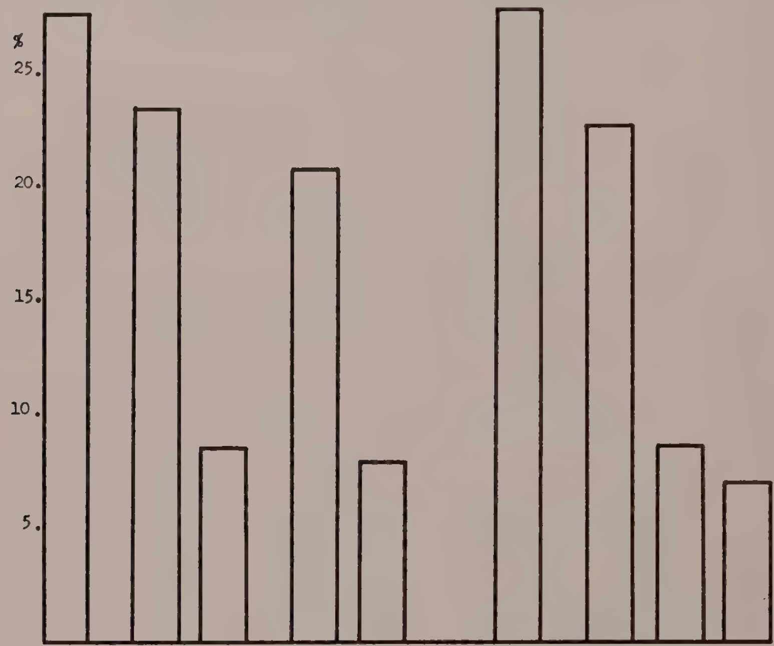
Group II: Given 10 γ of colchicine.

- (a) Colchicine only, no period of anoxia.
- (b) Anoxia for one hour followed immediately by colchicine; a second hour of anoxia 12 hours later.
- (c) Anoxia for one hour followed immediately by colchicine; additional one hour periods of anoxia at 6, 12, and 18 hours after injection.
- (d) Colchicine followed immediately by one hour of anoxia with additional one hour periods of anoxia at 6, 12, and 18 hours after injection.

X-irradiation was given with a Picker Industrial unit. The x-ray factors were 0.5 mm parabolic Cu plus 1.0 mm Al filters, H.V.L. 2.1 mm Cu. Roentgens measured in air. Colchicine was injected into the tail.

Anoxia was produced by placing the tadpoles in a shallow, covered, plastic box filled to overflowing with water which had been boiled 30 minutes. A constant environmental temperature of 20°C. was maintained. The majority of the tadpoles were killed 24 hours after treatment, a few were kept 48 hours before sacrifice.

The kidneys (containing the hematopoietic tissue) were fixed in Bouin's fluid, sectioned at 5 μ, and stained with blood stains. In the sections the percentage of destruction and/or division was determined with the use of a Whipple grid by tallying 300–400 cells in each tadpole (Allen, Schjeide, Millard and Piccirillo, '53).



Order	1	2	3	4	5	6	7	8	9
1	-	-	-	ANOX	ANOX	-	ANOX	ANOX	-
2	X500	X500	X500	X500	X500	COLCH	COLCH	COLCH	COLCH
3	-	ANOX	ANOX	-	-	-	-	-	ANOX
4	-	-	ANOX	-	ANOX	-	-	ANOX	ANOX
5	-	ANOX	ANOX	ANOX	ANOX	-	ANOX	ANOX	ANOX
6	-	-	ANOX	-	ANOX	-	-	ANOX	ANOX
	(47)	(19)	(18)	(13)	(21)	(44)	(14)	(18)	(8)

Fig. 1 Graph showing percentage of destruction and mitosis arrest of the hematopoietic cells of Bufo boreas halophilus treated with 500 r x-irradiation or 10 γ of colchicine as indicated. All counts made at the end of 24 hours. Abbreviations below columns indicate order of treatment.

Key to terms of diagram.

1. Anoxia 1 hr. before 500 r x-irradiation or colchicine 10 γ.
2. X-ray 500 r of colchicine 10 γ.
3. Anoxia (1 hr.) immediately after No. 2.
4. Anoxia (1 hr.) at 6 hr. after No. 2.
5. Anoxia (1 hr.) at 12 hr. after No. 2.
6. Anoxia (1 hr.) at 18 hr. after No. 2.

RESULTS

Results are given in figure 1. Five hundred roentgens of x-irradiation (column 1), and injection of 10 γ of colchicine (column 6) produce almost identical percentages of destruction of the hematopoietic cells 24 hours after treatment. The irradiation results in pyknosis; the colchicine produces recognizable arrests in the late prophase stage as well as pyknosis, which is often associated with such arrests.

Anoxia reduced the percentages of cell breakdown following x-irradiation and equally reduced the mitotic arrests in those tadpoles given 10 γ of colchicine. The delay in breakdown or mitotic arrest was much greater when 4 one-hour treatments of anoxia were given following irradiation or colchicine (columns 3, 5, 8, and 9) than when two one-hour treatments were given (columns 2, 4, and 7). Anoxia applied immediately after either x-irradiation or colchicine (columns 2, 3, and 9) had almost as great an effect in delaying the initial cell breakdown as when applied before and during treatment (columns 4, 5, 7, and 8): the differences between the groups receiving anoxia before and the groups receiving anoxia after such treatment are not significant.

DISCUSSION AND CONCLUSIONS

A comparison of the groups represented by columns 4 and 7 with those represented by columns 5 and 8 shows that the rate of cell breakdown, and to an equal degree, the rate of colchicine arrest, is roughly proportional to the total period of partial anoxia. These facts indicate that mitosis is slowed in rough proportion to the duration of partial anoxia in the tadpole.

To determine whether all those cells arrested by anoxia would eventually break down if given sufficient time following their anoxia treatment, 5 tadpoles from Group I (b) — x-irradiation plus two one-hour treatments of anoxia — were killed 48 hours after irradiation rather than at 24 hours. These animals, therefore, had a total recovery period following their last anoxia treatment of 36 hours. Destruction of their hematopoietic tissues was equal to that in animals receiving 500 r of x-irradiation without anoxia treatment.

From these facts, it is apparent that the action of post-irradiation anoxia in these tissues is to retard the rate of breakdown following 500 r x-irradiation rather than to protect the cells from eventual disintegration. As the irradiated cells are released from the influence of anoxia, they apparently proceed to the point at which they would normally divide, whereupon, the latent injury disturbs the cellular mechanism to the extent that the cells break down.

We conclude that anoxia, like low temperature, acts as an agent to slow down metabolic processes, and as such, delays the process of destruction.

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We call attention to the fact that the heavy irradiation and short time limit necessary in this experiment probably make it inapplicable to the problem of the protective influence of anoxia against irradiation damage.

BLOCKAGE OF IMPULSES IN THE DORSAL ROOT GANGLION BY CURARE

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Since the pioneer work of Bernard (1849–1850), which appeared a century ago, curare has been regarded as a drug which affects neither nerve nor muscle fibers. The blockage of neuromuscular transmission was explained by the assumption of an intermediate third substance necessary to the conduction of impulses from motor nerve to muscle, which was rendered ineffective by the administration of curare. This third element is identified in the current humoural theory of neuromuscular transmission as ACh, and curare is supposed to block transmission by occupying competitively the ACh receptors on the motor end-plate.

In a series of experiment [1] it was found that afferent impulses can be blocked in a dorsal root ganglion, if the preparation is treated with curarized Ringer's solution (1/5000 high potency curare, Merck; or 3×10^{-4} d-Tubocurarine chloride, Burroughs Wellcome). The

[1] Part of the experiment was carried out at the Department of Physiology, University of Otago Medical School, Dunedin, N. Z., 1951.

blockage is reversible (taking 3–4 hrs.) and can be demonstrated repeatedly in a single preparation. This is interesting because the sensory fibers are not synaptically interrupted in the dorsal root ganglion, and consequently there can be no "intermediate third substance" present.

Figure 1 shows the results of one such experiment. The root-ganglion-nerve preparation of the frog was used. The peroneal and tibial nerves were dissected out with the 9th ganglion and its roots. The nerves were stimulated together. The recording reference electrode was attached to the crushed central end of the dorsal root and the detecting electrode was put either proximal or distal to the gang-

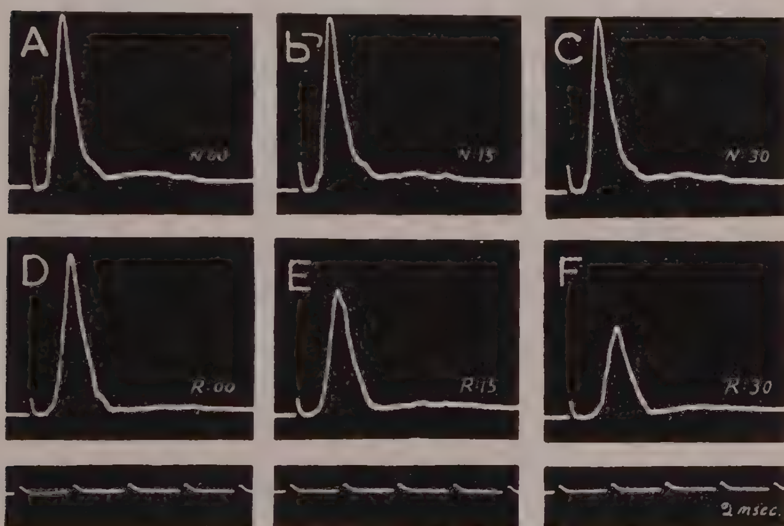

Figure 1

lion. For *A, B* and *C* only axons lay between the recording and stimulating electrodes. For *D, E* and *F* the dorsal root ganglion as well was included. *A* and *D* were taken immediately before the application of curare, *B* and *E* after 15 min., and *C* and *F* after 30 min. of curarization. The potential changes in the nerve trunk *(A, B, C)* remained unchanged, but those in the dorsal root *(D, E, F)* decreased to about half size. Because similar results were not observable with the ventral root, it is concluded that some of the sensory impulses must have been blocked in the dorsal root ganglion and most probably at the T-shaped bifurcation points (Dun, '51, '52).

With two stimuli applied at various short time intervals, the initial blocked impulses conditioned the site of blockage in such a way

that a second impulse passed through it. The plotted curve of facilitation resembled that found by Bremer and Homès ('30) at curarized neuromuscular junctions.

The similarity of curare effect at sensory ganglion and at neuromuscular junction would seem to demand a common explanation. The two structures are not known to have a common mechanism for chemical transmission on which curare could act. On the other hand, both structures involve branching fibers, believed to be critical points for impulse conduction. The question arises, may not the curare blockage at neuromuscular junctions be in part due to failure of conduction of impulses at the terminal branchings of motor nerve fibers? The fineness of the end branches and their lack of nerve sheath, perineurium and myeline sheath would enhance their sensitivity to curare.

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SUPPLEMENT

SOCIETY OF GENERAL PHYSIOLOGISTS

Abstracts of papers scheduled for the ninth Annual Meeting,[1] Marine Biological Laboratory, Woods Hole, Massachusetts, September 8 and 9, 1954, and for the sectional meeting of the Society with the A.A.A.S. and Western Society of Naturalists, Berkeley, Calif., December 27, 1954.

[1] Abstracts or summaries of programs of previous meetings will be found in Biol. Bull., *91:* 236; *93:* 222; *95:* 281; *97:* 267; *99:* 308 (1946–50); Science, *114:* 699 (1951) and *118:* 768 (1953); and J. Nat. Cancer Inst., *13:* 1379 (1953).

SYMPOSIUM

ELECTROLYTES IN BIOLOGICAL SYSTEMS

(Organized by A. M. Shanes, and dedicated to W. J. V. Osterhout, and M. H. Jacobs.) Woods Hole, September 8.

ABSTRACTS

1. PERMEABILITY OF MICROORGANISMS TO INORGANIC IONS, AMINO ACIDS, AND PEPTIDES. Dean B. Cowie and Richard B. Roberts, Carnegie Institution of Washington.

Escherichia coli is highly permeable to many materials. The materials of the medium diffuse into and out of the "water space" of the cell. The water space is that part of the total cell volume which has the same concentration of diffusible materials as is found in the medium.

With the use of isotopic tracers this space has been quantitatively measured and corresponds roughly to the actual water content of the cells, 70 to 80% of their wet weight. Direct measurements consist of immersing cells in media containing labeled compounds. After centrifugation, if no metabolic incorporation has occurred, the radioactivity of the cell pellet, per gram wet weight, is equal to the radioactivity of 0.75 ml of the immersion fluid. This radioactivity may be washed out of the pellet by simple dilution with nonradioactive medium and the amount of radioactivity removed is always proportional to the volume of dilution fluid. It is often necessary, however, to distinguish between metabolic incorporation and the quantity of diffusible materials held passively within the pellet. When such distinction is made, the same water space was observed for Na^+, K^+, Rb^+, Cs^+, Mn^{++}, $SO_4^{=}$, PO_4 , glucose-1-phosphate, fructose-1: 6-phosphate, cystine, glutamate, methionine and glutathione. When labeled proteins were used, an intercellular fluid space of 10 to 20% of the pellet volume was obtained.

The isotopic competition method also demonstrates cellular permeability. With the use of this method an exogenous C^{12}-amino acid may eliminate or markedly suppress, in intact cells, the formation of that particular amino acid from C^{14}-glucose. In reciprocal experiments, uniformly C^{14}-labeled amino acids, supplementing medium containing C^{12}-glucose, are found in proteins with the same specific radioactivity ($\pm$ 10%) as the exogenous compound. The intact carbon chain is metabolically incorporated demonstrating that penetration of the cell membrane occurred. Most amino acids found in bacterial protein, as well as nucleic acid bases, have been shown to be effective competitors. Similar results are obtained with *Torulopsis utilis* and *Neurospora crassa*.

The *E. coli* membrane is a morphological boundary within which are assembled most of the reactive centers of metabolism. The protoplasm of the cell is in direct

contact with the environment and may be likened to a sponge; the cell membrane to a surrounding hair net unable to exclude the entrance or emergence of small molecules. The concept of such a permeable membrane poses certain problems, however. During synthesis, the intermediates of the cell are not lost by diffusion and consequently some mechanism of retention must operate. Evidence in particular cases for mechanisms of binding will be discussed.

2. SODIUM AND POTASSIUM REGULATION IN ULVA LACTUCA AND VALONIA MACROPHYSA. George T. Scott and Hugh R. Hayward, Oberlin College, the Marine Biological Laboratory, the Bermuda Biological Station and the Woods Hole Oceanographic Institution.

Investigations on factors controlling sodium and potassium ion distribution in the cells of the marine green alga *Ulva lactuca* indicate: (1) A complete exchange of cellular for environmental K^+ with increased rate under illumination or increased temperature. (2) The presence of 10^{-3}M iodoacetate (IA^-) in the dark causes a marked loss of K^+ and gain of Na^+, phenomena which do not occur when the alga is illuminated. (3) The presence of 5×10^{-4}M AsO_4 completely protects the cells against the K^+ loss caused by 10^{-3}M iodoacetate in the dark but not against Na^+ gain resulting from IA^- inhibition. The K^+ protection by AsO_4 is understandable in terms of arsenolysis at the level of triose-phosphate-dehydrogenase, the site of IA^- inhibition. (4) Phenyl-urethane causes a loss of K^+ and gain of Na^+, changes which are completely reversible on washing the alga in running sea water under illumination. (5) The ''decoupling agent'' 4,6,-dinitro-o-cresol causes a marked progressive increase in cellular Na^+ and a drop in cellular K^+. (6) Pyruvate (50 mg %) given with IA^- (2×10^{-3}M) for 5 hours in the dark completely prevents the Na^+ increase caused by IA^- while affording less protection against K^+ loss. Phosphoglycerate, on the other hand, offers more protection against K^+ loss and essentially none against Na^+ gain. (7) Cells washed in isotonic sucrose after having lost 80–90% of their normal K^+ and Na^+, will reaccumulate K^+ linearly when returned to sea water. The rate of reaccumulation is independent of Na^+ in the medium (isotonicity maintained with sucrose), indicating, that K^+ accumulation is not secondary to Na^+ secretion.

Investigations on the large coenocytic alga *Valonia macrophysa* indicate: (1) The effect of iodoacetate (0.1–0.3M) in causing a loss of K^+ and gain of Na^+ is completely prevented by light. (2) At 2°–5°C. for 75 hours Valonia loses K^+ and gains Na^+. On transfer to sea water at 18°C. K^+ is slowly reaccumulated and Na^+ secreted for about 300 hours. (3) Phenyl-urethane (10^{-3}M) effects a marked loss of K^+ and a gain of Na^+ by the cell which is reversible on transfer to running sea water without the inhibitor.

Two major points are emphasized: (1) the close correlation between cellular metabolism and normal cation regulation; (2) the need for postulating separate mechanisms for transporting K^+ inwards and Na^+ outwards because of the differential influence of many of the above agents toward K^+ and Na^+ and the different kinetics in the ion movements.

3. THE RELATIONSHIP OF CELL SURFACE ENZYMES TO ION TRANSPORT IN YEAST CELLS. Aser Rothstein, University of Rochester.[1]

In the cytoplasm of the yeast cell, the principal cation is potassium, the principal anions are bicarbonate and phosphate. Under any conditions the yeast cell is relatively impermeable to anions and in the resting anaerobic state (no substrate) to cations. If substrate is added the cell apparently becomes permeable to monovalent cations, particularly potassium. Not only does the cell become permeable to potassium, but there is a net accumulation of this cation in exchange for hydrogen ions secreted from the cell; both ions move against activity gradients as high as 1000 to 1. The exact source of the energy necessary to drive the exchange of potassium and hydrogen ions is not known. The energy can be derived from anaerobic or aerobic metabolism of a variety of substrates. The processes can be blocked by a number of inhibitors. The specific source of the hydrogen ion is also unknown, although it is ultimately derived from the substrate.

A working theory can be constructed from the available data. It is suggested that there is an outer zone of the cell separated from the environment by an anion impermeable membrane and from the interior by a cation and anion impermeable membrane. The outer zone contains enzymes which are concerned in the metabolism of certain exogenous substrates. The cations of the medium can readily equilibrate across the outer membrane. Therefore, the rates of the reactions in the outer zone are influenced by the concentrations of potassium and hydrogen ions in the external environment, rather than those of the internal cytoplasm. Excessive extracellular concentrations of hydrogen ions inhibit metabolic reactions in the outer zone. Potassium reverses the inhibition in a competitive manner. In contrast, reactions of interior of the cell are relatively unaffected by extracellular potassium and hydrogen ions, being protected by the impermeable inner membrane.

The transport of potassium into the cell is accomplished by a metabolism-linked carrier system in the inner membrane. As the potassium is removed from the outer zone and carried into the interior of the cytoplasm, a further exchange of potassium ion and hydrogen ion is induced across the outer membrane. The immediate source of hydrogen ions may be a redox reaction in the outer zone. The ultimate source is the interior of the cell which becomes somewhat more alkaline as potassium is accumulated. There is no obligatory coupling between metabolic reactions of the outer zone and the mechanism for pumping potassium into the cell. Even at its greatest efficiency, only 1 molecule of potassium is pumped for each 10 molecules of glucose taken up.

[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, New York.

4. ELECTROLYTE TRANSPORT IN MITOCHONDRIA. Gilbert H. Mudge, Columbia University.

The mitochondrial fractions of homogenates of rabbit liver were isolated by differential centrifugation and then incubated under varying metabolic conditions. Exchange of electrolytes (sodium and potassium) between the mitochondria and incubation medium was examined with radioactive isotopes. Both before and after

incubation the mitochondrial fraction was washed in a solution free of the electrolyte under study, and the concentration of electrolyte and isotope were then directly determined both in the isolated mitochondrial fraction and in the incubation medium. When studied in this manner, the mitochondria are found to have a potassium concentration about three times greater than that of sodium. The metabolic activity of the mitochondria partially determines the concentration and rate of exchange of potassium, but not of sodium. Since the relatively slow rate of potassium exchange observed under these conditions is in contrast to the very rapid rate found by Bartley and Davies, this problem has been re-examined with particular emphasis on the conditions of the experiment, the manner of preparation and the concentration of various components, particularly potassium, in the incubation medium. These factors were found to play an enormous role. Hence any interpretation of the nature of mitochondrial electrolytes must at present be quite tentative, since all results are so overwhelmingly determined by the conditions of the experiment. Certain aspects of phosphate exchange and water metabolism will also be reviewed.

5. CATION TRANSPORT IN RED BLOOD CELLS. D. C. Tosteson, National Institutes of Health.

Experiments bearing on two aspects of the problem of potassium transport will be discussed. One concerns the diffusion of K across the red cell surface. The other involves the coupling of ion transport with cell metabolism.

Jacobs has shown that n-butyl alcohol markedly increases the permeability of the red cell surface to Na and K. Using K^{42} as a tracer, we have characterized the kinetics of K transport in human red blood cells exposed to butanol. The experiments were done at 26°C. on washed human red cells suspended in modified Ringer-phosphate medium containing added glucose at pH 7.5. In cells exposed to 0.2 M butanol, the K flux was, if anything, slightly less than that observed in the same cells incubated in the absence of butanol. In 0.3 M butanol, the inward rate constant for K transport was increased about five-fold while the outward rate constant was increased about 200-fold above the control values, so that the ratio of inward to outward rate constant approached unity. K influx was markedly increased when the K concentration in the medium was increased. These results are consistent with the idea that K transport in human red cells exposed to 0.3 M butanol is almost entirely by the process of diffusion.

Experiments bearing on the relation of K transport to cell metabolism were done on the nucleated red cells of the duck. The experiments were done at 37°C. on unwashed duck cells suspended in a glucose enriched, bicarbonate buffer at pH 7.5. Using K^{42} as a tracer, it was found that K flux in the steady state was about five times faster than in human red cells. K transport was about twice as fast in nitrogen as in 10% O_2. The cell K concentration was independent of the oxygen tension but cell water content increased 10% and cell metabolism converted from respiration to glycolysis upon removal of oxygen from the system. The acceleration of K transport by anoxia persisted for several hours in the absence of added glucose. However, if the cells were allowed to incubate in N_2 in the absence of substrate for 24 hours, the K flux was reduced to about 1% of the initial value. At this time the cell K concentration was about 80 mM/1.RBC.

These results, as well as the effects of various metabolic inhibitors will be discussed in relation to the mechanism of coupling of ion transport and cell metabolism.

6. FACTORS GOVERNING ION TRANSFER IN NERVE. A. M. Shanes, National Institutes of Health.

Ion transfer in nerve presents several distinct problems — those dealing with activity and those related to the ''resting'' state.

The electrical and chemical methods applied by English investigators to the ionic interchanges of activity have led to the most satisfactory quantitative description of the ionic events during the nerve impulse. Their conclusion that sodium entry largely precedes potassium exit leaves unanswered the question as to how this is achieved.

Both the steady state distribution of ions and the recovery from activity are dependent on metabolic processes. Thus, at lower temperatures potassium emerges from the resting axon and the reabsorption of potassium following activity is depressed. The dependence on metabolism is particularly evident from the sodium-potassium interchange resulting from metabolic inhibition and from the delay in this interchange by suitable substrates. Such experiments also reveal an important aspect of drug action, namely, the ability of depolarizing drugs such as veratrine, which elevate excitability, to accelerate ionic shifts and that of ''stabilizing'' blocking agents, like cocaine, to delay this exchange. These observations therefore provide a basis for relating metabolic processes to nerve electrochemistry as well as for elaborating mechanisms of drug action.

Results based on *net* transport usually do not permit isolation of the processes concerned with sodium and potassium transfer and cannot separate influxes from outfluxes, as may be done with radioisotopes. However, certain experiments reveal that sodium and potassium can move independently. For example, nerves which have gained sodium and lost potassium as a consequence of exposure to NaCl, recover potassium but fail to eject the excess sodium upon return to normal Ringer's.

Single large invertebrate nerve fibers have proven of great value, but deteriorate rapidly under *in vitro* conditions. The remarkable chemical, structural and functional stability of the desheathed sciatic of the toad, *Bufo marinus*, makes it particularly suitable for the study of the kinetics of penetration and of the steady state distribution of radioisotopes. An analysis of the results obtained with C^{14} labeled urea and sucrose and with $S^{35}O_4$, Cl^{36}, and Na^{22}, in the light of current literature, suggests (a) that the myelin sheaths contain an aqueous phase which is part of the ''extracellular'' space, (b) that substantial fractions of the nerve sodium and chloride are intra-axonal, and (c) that the meylin may play a part in ion exchange. The importance of nerve structure for these findings will be discussed.

7. ION TRANSPORT AND ION EXCHANGE IN FROG SKIN. Ernst G. Huf, Medical College of Virginia.

Since the earliest days of electrophysiology, frog skin has been a much favored object for studies which were devoted to the nature of bioelectric potentials and to the behavior of a living ''membrane'' towards the passage of ions, water and un-

charged molecules. Frog skin is also the seat of forces which act on ions, especially sodium ions, which are carried in the inward direction in as yet unknown metabolic reactions. ''Ion pump'' mechanisms are also at work in many other cells and tissues of the animal as well as plant kingdom. Despite its complex morphology, isolated frog skin, because of its ''membrane'' like structure and its relatively long survival time, lends itself to most any type of experiment designed to look into the physical and chemical aspects of active ion transport. Conventional chemical as well as tracer methods are applicable. The present report is not a complete bibliographical type of review, although a number of older papers on active ion transport in frog skin have been included. The emphasis is laid upon tying together the rapidly increasing frog skin literature under the headings: 1. Active fluid transport and one-way osmosis. 2. Active uptake of sodium chloride; factors in active uptake of sodium chloride and skin potential. 3. Movements of cations other than sodium ion in frog skin. 4. The ''passive'' and the ''active'' ion in salt transport. 5. Microscopic and submicroscopic structure of frog skin. 6. Mechanism of active cation transport; Conway's redox pump. 7. Dual pump hypothesis of active sodium transport in frog skin. Some of the author's as yet unpublished results on potassium in frog skin are briefly presented. It appears as if potassium in the skin is in a state of monolayer adsorption and that the dependence of adsorbed potassium on the external potassium ion concentration can be reasonably well described by applying stoichiometric considerations. Potassium in the skin may be exchanged for sodium in the bath. If this occurs, little or no sodium chloride is transported across the skin from the epithelial to the chorion side. Ion transport and ion exchange are closely related processes. Potassium seems to be one of the important physiological regulators of active sodium transport. Throughout the discussion the work of W. J. V. Osterhout and M. H. Jacobs is appreciated as being fundamental to the field of active transport.

Contributed papers

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8. THE INDUCTION OF ION TRANSPORT IN FROG SKIN. Bradley T. Scheer, J. R. Rowley and W. R. Fleming, University of Oregon.

When a frog skin membrane separates two small volumes of NaCl solution or Ringers, gradients of electric potential and pH are established well before any appreciable sodium or chloride movement. Calculation of chemical potentials shows that the electric and hydrogen ion gradients are always far in excess of the sodium or chloride gradients at any stage in transport. A simple model of the ion-transporting system in frog skin can be developed on the basis of Ussing's carrier theory, which will exhibit these same properties; the power for ion transport is provided in the model by a differential pattern of oxidation-reduction reactions. Using ordinary liquid junction potential theory, it is possible to calculate the theoretical gradient of chemical potential of sodium ion in the model at from 100 to 300 millivolts; the observed gradient of oxidation-reduction potential in frog skin is 200 millivolts. To this extent, the model appears to correspond with observed conditions in the skin.

9. The effect of sodium azide on the respiration and ionic content of frog nerve. W. P. Hurlbut and F. Brink, Jr., Rockefeller Institute for Medical Research.

An oxygen cathode flow respirometer (Carlson et al., '50) was used to measure the steady state rates of oxygen consumption of frog *(R. pipiens)* sciatic nerve in various concentrations of sodium azide (0.2–5 mM). Flame spectrophotometric analyses were made of the sodium and potassium contents of nerves that had been soaked in these azide concentrations.

Inhibition of the resting respiration of frog nerve results in a loss of potassium and a gain of sodium and water. The potassium loss occurs with all concentrations of azide that inhibit the resting respiration. The rate of potassium loss increases with the azide concentration, and once leakage has been initiated, it continues at a relatively constant rate for as long as 12 hours.

Some evidence indicates that the increased weight of the poisoned nerves represents an increase in the quantity of extracellular Ringer's solution. Assuming this to be the case, the tentative conclusion is reached that sodium and potassium ions do not always exchange in equal quantities across the nerve fiber surfaces.

Stimulation of frog nerve at a rate of 50 volleys per second for from three to 5 hours results in a loss of potassium and a gain of sodium. These changes are slowly reversed during subsequent periods of rest. Two-tenths millimolar azide has no substantial effect on the net ionic shifts that accompany activity in frog nerve. This concentration of azide has little effect on the resting respiration of frog nerve but almost completely eliminates the increase of oxygen consumption that accompanies activity in the nerve (Brink et al., '52, and Doty and Gerard, '50).

10. Uptake of phosphorus by irradiated and nonirradiated yeast. A. B. Almeida and F. G. Sherman, Brown University.

Washed suspensions of *Saccharomyces cerevisiae* were aerated in phosphate buffer at pH 7 for 18–24 hours. Starved cell suspensions were irradiated with 200 kv X-rays (60,000 r). Immediately after irradiation, aliquots of irradiated and nonirradiated cells were diluted with phosphate buffer containing 1% glucose and 2 μc of P^{32} (as inorganic PO_4)/ml. Samples were taken at intervals for the determination of total cell activity, specific activity of inorganic ortho phosphate, total acid soluble phosphate, RNA-phosphate and DNA-phosphate. Total RNA and DNA/cell were also determined. Eighteen hours after irradiation the cells were washed and resuspended in nonradioactive phosphate buffer. The activity of P^{32} in the cells and medium was determined at hourly intervals after resuspension in radioactive free medium.

During the first 15 minutes after irradiation, the irradiated cells take up more inorganic P^{32}/cell than do nonirradiated cells and continue to do so for at least the first three hours after irradiation. The increase in P^{32} is found in the total cell activity (cts/min/mg dry wt), inorganic ortho phosphate, total acid soluble phosphate and the RNA-phosphate fractions. Specific activity of DNA-phosphate is reduced in the irradiated cells after the first hour following irradiation. The total amount of RNA/cell appears to increase slightly in irradiated cells during the first three hours after irradiation. There was no significant change in the amount

of DNA extractable in either the nonirradiated or irradiated cells. Neither irradiated nor nonirradiated cells lost P^{32} when they were suspended in nonradioactive PO_4 buffer.

11. THE DRIVING FORCES BEHIND THE ELECTROLYTE TRANSPORT IN BIOLOGICAL SYSTEMS. Reinhard H. Beutner, Des Moines Still College.[1]

Hodgkins' and Huxley's important discovery of the "overshoot" has pulled the props from under Bernstein's theory, which is now sustained by new hypotheses such as the Na migration and the fabulous Na pump. Bernstein neglects the contact potential differences at the membrane's phase boundaries, both inside and outside, working in opposite directions. A feature of these contact p.d.'s is that they are influenced by low concentrations of chlorides of fat-soluble organic bases such as choline or acetylcholine. Choline is contained in phospholipids bound chemically. Liberation of the electrogenic choline may be the cause of the up-stroke of the spike. Decomposition, once set going, is bound to extend throughout the membrane, and upon reaching the opposite membrane contact surface causes the spike down-stroke. Thus, a single reaction causes both up- and down-stroke. Evidence for this new concept is based on direct observations on nerve and muscle. Agents accelerating phospholipid splitting should shorten the down-stroke. Cholinergic drugs are such agents (Hokin and Hokin); and indeed they do shorten the down-stroke (Hoffman and Suckling). (According to Bernstein and Nachmannsohn acetylcholine should render membranes more permeable, *and* the down-stroke should be caused by decreased permeability, hence, be extended, whereas it is actually shortened.) According to our concept, cholinergic blocking agents should extend the down-stroke. This is confirmed by recent experiments. All facts of Hodgkins and Huxley fit well into this new concept.

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[1] This investigation was supported by research grant H-1861 from the National Heart Institute of the National Institutes of Health, Public Health Service.

12. THE PERMEABILITY OF RAT LIVER MITOCHONDRIA TO NON-ELECTROLYTES. Henry Tedeschi and Daniel L. Harris, University of Chicago.

The presence of a lipid or lipoprotein surface on the mitochondria has long been postulated on the basis of composition and staining properties (Cowdry, '26; Bourne, '35; Bensley, '37).

The osmotic sensitivity of mitochondrial enzymes, the characteristic swelling and shrinking of mitochondria in anisotonic media, as well as the release of large molecules from mitochondria at rates dependent on the nature of the medium have led several authors to infer the presence of a semipermeable membrane. (cf. Lehninger, '51; Schneider and Hogeboom, '51; Berthet et al., '52; Schneider, '53). Recent electron micrographs conclusively demonstrate the presence of a morphological membrane (Palade, '52, '53; Sjostrand and Rodhin, '53).

The classical photoelectric methods of following swelling and lysis of red blood cells have been applied to sarcosomes of the rat heart (Cleland, '52). A similar method has been utilized in the present study of rat liver mitochondria.

Liver from a rat starved for 15–20 hours was homogenized at 2–5°C. in a medium consisting of .25 M sucrose, .02 M potassium phosphate and .02 M sodium

versenate at pH 7.5. Following a preliminary centrifugation at 900 g, the particles were isolated from the supernatant by centrifugation at 8,500 g. They were then resuspended in fresh medium.

The mitochondrial suspension was exposed to a large volume of non-electrolyte solutions (.3 M, with .02 potassium phosphate, pH 7.5). The changes in optical density were measured at a wavelength of 520 mμ. The time required to reach a suitable optical density as end point, was taken as a measurement of permeability.

The results to be reported indicate the mitochondria possess a semipermeable membrane, lipid in nature and somewhat similar in properties to the cell membrane.

13. Re-hemolytic characteristics of human erythrocyte ghosts and the mechanism of hemolysis. Joseph F. Hoffman, Princeton University.

When ghosts of human erythrocytes are exposed to hypotonic solutions or to lytic agents they can undergo further hemolyses. This process of re-hemolysis of ghosts has been found to be quite similar to the hemolysis of intact red cells. For example, the ghosts swell prior to re-hemolysis and this re-hemolysis is all-or-none. During re-hemolysis the hemoglobin (Hb) concentration became equal inside and outside the ghosts. After re-hemolysis had occurred the ghosts again became impermeable to Hb. However, Hb was found to *enter* the ghosts at the time of re-hemolysis if the concentration of Hb in the medium was greater than that in the ghosts.

Ghosts containing various fractions of their original Hb were prepared by appropriately altering the volume of hemolyzing solution relative to the volume of intact cells. When washed and suspended in 0.17 M NaCl - PO_4 buffered media, the ghosts returned to their initial volume and the rate of re-hemolysis was observed to be proportional to the concentration of Hb in the ghosts. As with intact cells, the rate of re-hemolysis was accelerated by the addition of n-butyl alcohol (BA). For a given concentration of BA, temperature and Hb content, the rate of re-hemolysis was minimal around the isoelectric point of Hb. The shapes of the re-hemolysis curves appear identical to that of intact cell populations. Re-hemolysis by BA was inhibited by the addition of sucrose to the medium. These results on the re-hemolytic characteristics of ghosts are consistent with the colloid osmotic theory of hemolysis.

14. Experiments on mosaic membranes with cation-permeable and anion-permeable parts. Rex Neihof, National Institutes of Health.

The rate of diffusion of electrolyte across mosaic membranes which are composed of selectively anion-permeable (cation-impermeable) parts and selectively cation-permeable (anion-impermeable) parts in juxtaposition is one of the few complex membrane processes which has been subjected to a quantitative, theoretical analysis (Sollner, '32).

The theoretical model consists of a cyclic arrangement of four component parts: dilute solution/anion-selective membrane/concentrated solution/cation-selective membrane/dilute solution. In such a system cations move from the concentrated to the dilute solution across the cation-selective membrane and an equivalent number of anions move through the anion-permeable membrane. The movement of ions

corresponds to a flow of current in the system, the two phenomena being two different aspects of the same physical process. The number of electrochemical equivalents of electrolyte which penetrate across the membranes in the model system must be identical with the number of faradays of electricity which flow during the same period.

The general electrochemical considerations are presented which form the basis for an experimental test of this theory with all-electrolytic model systems as well as with models which involve the use of auxiliary electrodes. The number of faradays moved and the number of electrochemical equivalents of electrolyte translocated on closed circuit show a 1: 1 ratio with a mean deviation of less than $\pm$ 2.5% under a variety of experimental conditions. The predictions of the theory must be considered as proven quantitatively well within the limits of the experimental error.

15 THE POTASSIUM UPTAKE AND OXYGEN CONSUMPTION OF FROG MUSCLES IN THE PRESENCE OF INSULIN AND LACTATE. J. F. Manery, L. B. Smillie and K. E. Toye, University of Toronto.

The rate of oxygen consumption and the uptake of potassium by intact frog muscles, in the presence of insulin and lactate, were studied at three different potassium concentrations: 2, 6 and 10 m. eq. per liter of Ringer's solution. The normal rate of oxygen consumption in Ringer's solutions containing 2 and 6 m. eq. of K per liter was 35, in that containing 10 m. eq. of K per liter was 130 mm^3 per gram per hour. At the lowest potassium concentration insulin stimulated the oxygen consumption, while at the highest concentration of potassium insulin caused a depression of the oxygen consumption.

The uptake of potassium due to the presence of insulin occurred at all three of the potassium concentrations studied, that at 6 and 10 being larger than that at 2 m. eq. of K per liter of Ringer's solution. At 6 m. eq. of K per liter the average potassium uptake was 11.66 $\pm$ 0.82 m. eq. per kilogram of muscle and this occurred in about 4 hours after the addition of insulin and lactate.

Both dinitrophenol and monoiodoacetate abolished the insulin-stimulated potassium uptake. Glycogen determinations were performed on the muscles at the termination of the experiments.

16. MEMBRANE EXCITATORY PHENOMENA AND OLFACTION. L. J. Mullins, Purdue University.

For many common solvents, the threshold for olfactory perception is about 10^{-3} of the vapor pressure of the substance. In homologous series of paraffins, chloroparaffins, or alcohols, the threshold declines from C_1–C_4, and then rises from C_4–C_{13}. Unsaturated hydrocarbons with certain steric configurations are very much better olfactory stimulants than are other unsaturated hydrocarbons. This observation suggests that it is not the chemical reactivity of the compound but rather the shape of its molecules that is of importance in causing olfactory effects. From the observation that both the alcohols and the paraffins are about equally effective in olfactory stimulation, whereas they differ in their abilities to cause narcosis, it is possible to infer that these substances act on different limiting receptors. Adapta-

tion to continuous chemical stimuli apparently takes place because of a competition between two processes: excitation and narcosis. It is suggested that these two physiological states correspond to ''leaky'' and ''non-leaky'' membranes.

17 MECHANICAL PROPERTIES OF ORGANIZED COMPONENTS OF SURFACE-SPREAD ACTOMYOSIN FIBERS. Teru Hayashi and Raja Rosenbluth, Columbia University and Marine Biological Laboratory.

Fibers formed of surface-spread actomyosin (''pellicular fibers'') are essentially organized macromolecular aggregates. Earlier experiments had shown these fibers to respond to mechanical stress in a manner that could be explained if it were assumed that within the fibers there were, functionally speaking, component ''links'' and ''springs'' in a series arrangement. From these early experiments, it was inferred that these components must have certain physical properties underlying the total behavior of this heterogeneous system.

Using the method of stretches and releases under isotonic and isometric conditions, it has been found that some degree of separation of the action of these components is possible in the elucidation of their physical properties. The experimental results show that the ''springs'' are elastic in the sense that deformation due to stretch is reversible, so that it becomes possible to calculate an ''extensibility modulus'' for this component alone. Likewise, it can be shown that such agents as ATP, salts, and temperature do not affect these elastic components. The ''links,'' on the other hand, appear to be plastic. The decay of tension after stretch of the fiber is primarly due to the plastic elongation of this component. The time characteristic of this process appears to be a complex one, in which two components seem to be involved.

18. SIDEROPHILIN, IRON-BOUND AND IRON-FREE, AND THE TOTAL IRON-BINDING CAPACITY OF SERUM OR PLASMA. Arthur L. Schade, National Institutes of Health.

New, rapid, and reliable methods for the determination of siderophilin-bound iron and of iron-free siderophilin in small amounts of sera or plasmas, whether ''hemolyzed,'' icteric, or high or low in bound iron, have been devised.

Bound serum iron is determined by adjustment of the serum sample with concentrated phosphate buffer to a pH value at which the constituent proteins remain in solution but the siderophilin-bound iron is wholly dissociated and available to combine with a suitable chromogenic agent, in this case terpyridine. Ascorbic acid is used as the iron reductant. The iron-terpyridine complex in the treated serum is measured by light absorption of a test solution at λ 552 mμ against a treated serum control minus terpyridine. A standard iron-terpyridine curve permits ready estimation of the amount of iron in the original serum sample.

The proposed method for the determination of the iron-free siderophilin, or the unsaturated iron-binding capacity of the serum (U.I.B.C.), involves the addition to serum of iron in excess of that capable of being bound, followed by direct analysis of the serum sample with terpyridine as the chromogenic agent without acidification or the removal of the constituent proteins. A serum control to which the same amount of iron but no terpyridine is added furnishes the necessary correction for

the absorption at λ 552 mμ of the iron-siderophilin complex in the test sample. The difference between the quantity of iron added to the serum and that found to be in excess is equal to the U.I.B.C.

19. SOME NATURALLY OCCURRING ANTIMITOTIC SUBSTANCES. L. V. Heilbrunn, University of Pennsylvania.[1]

All the diverse agents which induce egg cells to divide cause a gelation in the protoplasm; this gelation is the result of a clotting reaction similar to that which occurs in blood. Thus, as might be expected, the mitotic gelation is prevented by heparin and similar substances. Heparin-like substances are present generally in living material and they help to maintain a colloidal balance in the protoplasm; these substances are especially abundant in ovaries and they are also given off by muscle when it is stimulated. Tests were made on marine eggs, especially eggs of the worm *Chaetopterus*. Substances which maintain the protoplasm of these eggs in a fluid state and which also prevent mitosis were obtained from the ovaries of 14 species of fishes, 2 amphibians, a bird and 3 mammals; likewise from lobster muscles and from frog muscles. Chemical studies are now in progress to determine insofar as possible the chemical nature of these substances on ascites tumors of the mouse, and our preliminary results are encouraging.

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health.

20. SENSORY MECHANISMS OF INSECT SPIRACLES. James F. Case, Army Chemical Center.

The nature and manner of transmission of the stimuli causing opening of the spiracles of insects are discussed. Oxygen pressure was found to modify the effects of CO_2 on spiracle opening in the housefly, *Musca domestica*. As the pO_2 is increased from 34 to 400 mm Hg the median effective concentration of CO_2 for spiracle opening rises from 26 to 108 mm Hg, as expressed in the equation $\log ED_{50} = .605 (\log pO_2) + .097$. The spiracular response to CO_2 is shown to be adaptive.

The specificity of the opening stimulus was investigated by exposing houseflies to irritant vapors and by injection experiments. Only those compounds producing acidosis were able directly to elicit spiracle opening. Localized injections of lactic acid in fleshflies, *Sarcophaga bullata*, were shown to produce opening of the nearest spiracle indicating considerable independence of response.. Thoracic spiracle function is, however, dependent upon intact connections with the thoracic ganglion.

21. HYDRAULIC AMPLIFICATION OF SENSITIVITY IN FREE AND FIXED VOLUME MANOMETRY. George Hobby and Dean Burk, National Cancer Institute and Max Planck Institute for Cell Physiology.

Amplification of any given, usual sensitivity of the conventional Warburg manometer up to 10 to 100 times can be accomplished in both constant volume and free manometry, without any necessary structural alteration in the manometer, by suitable application of the hydraulic principle that linear displacement of a given volume of fluid in an enclosed tube is a function of the area of the tube at any

given point or section. The sensitivity magnification is effected in practice simply by raising the level of the manometric fluid from its usual position in the open arm capillary up into the expanded reservoir section atop the capillary, and inserting a small stable air bubble into the capillary by means of e.g. a long hypodermic needle. The linear traverse H of the moving boundary bubble in the capillary of area a is greatly magnified compared to the linear traverse h of the fluid meniscus in the reservoir of area A, and is an inverse function of the ratio of the two respective areas. In fixed volume manometry the theoretical sensitivity magnification $M = A/a = H/h = k/K$, so that $H = hM$ and $K = k/M$, where k is the standard vessel constant parameter $[(v_G 273/T + v_F \alpha)/P_o]$, and K the vessel constant with magnification, such that x mm^3 gas change $= hk = HK$. In free manometry, where the confining fluid in the closed arm is not maintained at a constant level but is allowed to move freely against constant external pressure, $M = \bar{H}/h = k/\bar{K}$, where $\bar{K}$ is the vessel constant in free manometry $[\cong k(a/A + a/a^*) + a273/T]$, a^* is the area of the section of the closed arm where the manometer fluid confines the gas phase, and $\bar{H}$ is the linear displacement of the bubble in the open arm, such that $x = hk = \overline{HK}$, and $\bar{H} = hM$ and $\bar{K} = k/M$, for a given value of x and a given value of k. Attainment of maximum sensitivity magnification obviously requires, in addition to the reservoir-bubble arrangement, that the open arm capillary area a be small ($\sim 0.05 - 0.1$ mm^2) compared to both a^* and A so that in the limit $M \cong k/a \cong (0.5 \text{ to } 5)/0.05 \cong 10 \text{ to } 100$. For details, see Science, *120:* 640 (1954).

22. A PHYSIOLOGICAL CRITIQUE OF THE GILBERTS' GLACIAL INTEGRATION THEORY. Elisio Jesus Coraje, International Board of Hygiene.

(No abstract received. A delayed and somewhat cryptic telegram from Point Barrow suggests that Professor Coraje had been cut off by a glacier in the remote Brooks Range.)

23. RESPIRATORY METABOLISM OF MAMMALIAN CELLS PROPAGATED IN TISSUE CULTURE. Kenneth C. Fisher, University of Toronto.

The cells used in this study were Earle's L strain cells which were derived from the subcutaneous connective tissue of a normal, adult C_3H mouse. These cells have been propagated since 1940 in a medium consisting of horse serum, chick embryo extract and a balanced saline solution containing glucose.

For the experiments reported here the cells were suspended in a balanced saline solution that did not contain glucose. Cytochrome was not detectable in these cells by direct spectroscopic examination and the rate of oxygen consumption was unaffected by the concentrations of cyanide and azide that affect most cells. The addition of glucose or lactic acid to the saline solution did not affect the rate of oxygen consumption, although the cells rapidly form lactic acid from added glucose, as has been reported previously.

Conventional analytical procedures suggest that these cells do not contain appreciable quantities of glycogen. Estimations of the respiratory quotient by several different procedures yield values close to 0.7.

The rate of oxygen consumption appears to be relatively independent of the osmotic pressure, the sodium concentration and the potassium concentration of

the suspending medium. The rate is sensitive to pH, however, and is within limits higher at high pH levels.

It is a pleasure to acknowledge that the facilities provided by Dr. Raymond Parker made possible the propagation of the cells used here.

24. ELIMINATION OF THE EFFECTS OF TURBIDITY IN SPECTROPHOTOMETRY. J. B. Bateman and G. W. Monk, Camp Detrick.

Radiation is introduced through a small hole into a cavity with walls of high diffuse reflectivity. The radiation emerging from a small exit hole, measured by a photomultiplier tube, is extremely sensitive to the presence of absorbing substances within the cavity, while turbidity has a relatively insignificant effect upon the emergent radiation. The applicability of the device to biological systems is illustrated by the results of measurements of the absorption spectra of (a) intact erythrocytes and (b) suspensions of *E. coli,* over the wavelength range 375 to 600 mμ. In the former case the Soret band was clearly resolved. In the bacterial suspensions the bands of the oxidized and reduced cytochromes were accurately and sensitively recorded.

25. MECHANICAL PROPERTIES OF INSECT FLIGHT MUSCLE. Edward Boettiger, University of Connecticut and Edwin Furshpan, California Institute of Technology.

During flight in certain insects muscle action potentials and shortening are not associated. Experiments were performed to test the suggestion previously made by us (Biol. Bull., *99:* 346), that in these cases flight movements result from changes in tension produced by alternate quick release and quick stretch of fully tetanized antagonistic muscles. The longitudinal flight muscle of the bumble bee in tetanus gives up to 50 gm tension under isometric stimulation. Nearly maximal tension is produced at *in situ* length. No tension is recorded in muscle allowed to shorten less than 1.0 mm. A quick release of about 0.1 mm reduces tension nearly to zero. Tension falls rapidly during the release. A further small drop in tension usually occurs after release and is followed by a rise to the value characteristic of the new length. A quick stretch following a loss in tension by release results in a rapid rise in tension during the stretch followed by a slower rise to full tension. The rise in tension during the stretch may be as low as 25% of the total tension, 75% returning by active processes after the longer length is attained. Restretching at longer intervals after the release usually reduces the amount of the active rise. Under proper conditions a rise in tension above that occurring during the stretch can be obtained without previous release. A very rapid stretch, 1–2 msec., induces a large transient increase in tension, a fall to low tension and a slower active rise.

26. ON THE MECHANISM AND SIGNIFICANCE OF CYCLIC CO_2 RELEASE IN INSECTS. John Buck and Margaret Keister, National Institutes of Health.

Punt reported that certain insects release CO_2 in large brief "bursts" interspersed with much longer periods of low-level release. Schneiderman and Williams discovered that O_2 uptake nevertheless is level and continuous. We showed that the

burst cycle depends on normal spiracular activity. Burst production by spiracular regulation presents no serious problem, but the situation in interburst, during which (in diapausing pupae of the moth *Agapema)* over three O_2 molecules enter for every CO_2 molecule which escapes, is paradoxical, because any mechanical barrier to CO_2 escape, such as the spiracular valves, should also impede O_2 entry. A suggestion of Rubert Anderson's led to our computing diffusive transfer through an aperture just large enough not to limit O_2 uptake. Since apertural area and diffusion distance are the same for O_2 and CO_2, their relative transfer rates are, by Fick's first law, proportional to diffusivities $\times$ gradients. The O_2 gradient was shown to be effective, in causing differential diffusion, only in its final drop within the tissues, which, though small, is assumed *to be* larger than the outward gradient of CO_2. Additional confirmatory evidence is given by our measurements and computations of water loss and by Schneiderman and Williams' and our data on the effects of temperature, pO_2 and metabolic rate on CO_2 release. The CO_2 burst cycle is considered an incidental consequence of a mechanism functioning primarily in reducing transpiratory water loss.

Contributed papers

BERKELEY, MONDAY, DECEMBER 27

27. ACTION POTENTIALS IN SEA URCHIN EGGS.[1] B. T. Scheer, A. Monroy, M. Santangelo and G. Riccobono, Universitá di Palermo and University of Oregon.

The unfertilized eggs of *Paracentrotus lividus* and *Arbacia lixula* exhibit no difference in potential between a microelectrode filled with salt solution and inserted into the cytoplasm and a similar electrode of larger diameter in the external medium. There is likewise no resting potential difference in the fertilized egg. When the egg is fertilized, transient potential differences appear between the two electrodes, coincident with the visible changes in the egg cortex which accompany fertilization. Oscilloscopic recording shows the electrical changes to consist of a series of brief bursts of electrical activity of complex form, differing in the two species studied. The bursts are oscillatory in character, and from 5 to 15 millivolts in magnitude, with the internal electrode positive. As many as 50 bursts have been observed in a single egg, persisting only as long as the visible changes in the cortex.

[1] This work was done during tenure of a Fulbright Research Scholarship in Italy by the senior author, and supported by Fulbright Act funds administered by the American Commission for Cultural Exchange with Italy and by a grant from the Consiglio Nazionale delle Richerche.

28. GROWTH AND MACROMOLECULAR TRANSFER. Patricia F. Knight and A. M. Schechtman, University of California (L.A.).

The ovum of the bird passes through a relatively long period of slow growth until it reaches about 0.5 mm diameter. In the week following, the ovum amasses 85–99% of the final volume attained at the time of ovulation. Previous investigators have found that radiophosphorus, Sudan III, Newcastle disease virus, and arsenic compounds are detectable in ova which have reached the period of rapid growth. In the present experiments, rat serum, crystalline bovine albumin, bovine gamma-

globulin, and lobster serum were injected into the circulation of laying hens, and the yolks of eggs laid during the month following injection were tested serologically for the presence of the foreign substances. Clear precipitin reactions were obtained for each of the above antigens in the saline-soluble fraction of the yolks. Bovine albumin and gamma-globulin activities were studied more closely. Activity for these antigens appears in eggs laid 2–3 days after injection of the hen, reaches maximal titers at 5–8 days, and is absent from eggs laid 16 days or more after injection. The bovine activities seem to exist in livetin independent of the yolk proteins since the latter can be precipitated with anti-livetin, without reduction of titers for the bovine antigens. In paper electrophoresis, bovine albumin and gamma-globulin activities migrate with the mobility of the original protein preparations. The results are interpreted to indicate that foreign proteins may pass into the bird ovum in macromolecular form during the period of rapid growth.

29. Growth and protein stability: the serum proteins of the chick embryo. Werner G. Heim and A. M. Schechtman, University of California (L.A.).

The serum of the chick embryo is, in comparison with the adult, a very unstable system. At temperatures slightly above 0°C. certain constituents separate out to form a floating pellicle. After freezing-thawing and after lyophilization-reconstitution considerable quantities of insoluble material are formed. Under similar conditions adult sera show little (in the laying hen) or almost no perceptible denaturation.

Electrophoretic examination of sera from chicks at several stages between the 10th and 21st days of incubation show that the *proportions* of most of the components show no significant alteration as a result of freezing or lyophilization. The pre-albumin components, however, are significantly reduced. These components are characteristic of the embryonic serum and disappear within a few days after hatching. P^{32} injected into the egg white appears largely in the trichloroacetic acid soluble fraction of *fresh* chick serum. After lyophilization the denatured fraction of the serum contains a considerable amount of the P^{32} activity. The results indicate that the pre-albumin components (which are rich in P) are readily denatured and form part of the insoluble precipitates.

30. Colloidal stability in growing tissues: studies on hepatoma and normal liver. Arthur Hirata and A. M. Schechtman, University of California (L.A.).

Normal liver tissue from Irish rats and transplantable hepatoma originating in the same strain were homogenized in Lowry's intracellular salt solution and supernatants obtained by centrifugation at 15,000 $\times$ g for two and one-half hours.

The heat stabilities of these supernatants were tested at 58°C., the progress of aggregate formation being followed nephelometrically. Hepatoma consistently shows a significantly higher degree of stability. When the two tissue supernatants are brought to equal concentration, on the basis of absorption at 280 mμ or total nitrogen, the same differences are observed.

Electrophoretic analysis shows 4 major components in each of the supernatants. The second (next to fastest) component of the hepatoma supernatant shows a

marked increase relative to the same component of the normal liver. The first (fastest) components of both liver and hepatoma have mobilities exceeding that of serum albumin and their absorption spectra are characteristic of nucleic acids. It is considered probable that the differences in heat stability of the supernatants is due to some qualitative or quantitative differences in the 2nd, 3rd, or 4th electrophoretic components. The first component (nucleic acid or nucleoprotein) does not seem to be an important factor.

31. THE INDUCED FORMATION OF A NEW PEROXIDASE BY THE PLANT GROWTH HORMONE, INDOLEACETIC ACID. A. W. Galston and S. M. Siegel, California Institute of Technology.

It has previously been shown that the indoleacetic acid (IAA) oxidase of peas consists of a peroxide-producing flavoprotein coupled through H_2O_2 to a peroxidase which numbers IAA among its substrates. This IAA oxidase has been further demonstrated to be adaptively formed in response to IAA or other auxin molecules.

In the present work, the peroxidase has been shown to be the adaptive member of the complex. This peroxidase differs from conventional horse-radish root peroxidase (HRP) in at least the following respects: (1) It has an apparent molecular weight below 20,000 as contrasted with ca. 44,000 for HRP. (2) It is appreciably soluble in saturated ammonium sulfate. (3) It is much more active toward indole-containing compounds than HRP. (4) It is much more labile than HRP during storage in the cold or at room temperature, and (5) It is rapidly and completely inactivated by incubation with coenzyme A.

Induction of peroxidase proceeds optimally with ca. 10^{-7} M IAA, in cells of the elongating portion of root or stem. Embryonic or dividing cells are relatively insensitive, and well-differentiated cells do not respond at all. Induction is inhibited by oxygen deprivation or by ca. 10^{-4} M 2,4-dinitrophenol.

α-Naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid, both active auxins, are effective inducers of this peroxidase. Conventional peroxidase substrates, such as guaicol and eugenol are completely ineffective.

The implications of this work for the control of plant growth and for adaptive enzymology will be discussed.

32. MEASUREMENT AND HISTOCHEMICAL LOCALIZATION OF IAA-INDUCED PEROXIDASE ACTIVITY IN THE ROOT TIP OF VICIA FABA. William A. Jensen, California Institute of Technology.

The first 3 mm of the primary root of *Vicia faba* were analyzed morphologically and cell counts made. This area contains the root cap, general meristem, provascular tissue, protophloem and the early stages of the protoxylem. Peroxidase activity was measured in 200 μ sections before and after a two hour treatment with IAA at either 10^{-6}M or 10^{-7}M. Localization studies were made before and after similar treatment.

In the untreated root peroxidase activity is high in the sections containing the root cap and the forming protophloem tissue. Localization shows the activity con-

centrated in the root cap cells, the proepidermis, the forming protophloem and protoxylem. Little or no activity was found in the general meristem cells, the early provascular tissue or the cortex.

After two hours in 10^{-7}M IAA the peroxidase activity in all sections, except those containing the root cap and general meristem, was greatly increased. Localization showed the increased activity in the provascular tissues, protophloem and protoxylem. The general meristem cells were not affected and continued to show little activity. After similar treatment at 10^{-6}M IAA there was a general depression of peroxidase activity in all sections except those containing the root cap and general meristem.

33. MECHANISM OF TRANSFORMATION OF CHEMICAL INTO ELECTRICAL ENERGY IN THE TISSUES.[1] R. Beutner, Des Moines Still College.

The tools for this transformation are the ''living'' membranes which make up the electric battery system existing in tissues. A ''living'' membrane has chemical reactions occurring in it, while no such reactions occur in a ''dead'' or artificial membrane, such as used for osmotic experiments. Any membrane produces electromotive forces by dint of the electric potential differences located at the contact of the membrane with aqueous solutions, on *both sides* of the membrane. The membrane does not act as a sieve for ions, but, it conducts the current (1) by means of ions contained in it *a priori;* (2) if ions penetrate the membrane substance, they do so by a chemical exchange reaction, not merely by diffusion. Evidence for these facts will be submitted. The resting potential is, therefore, to be accounted for by an oxidative process which prevails more at the outer than at the inner membrane contact surface, since O_2 has more readily access to the outer than the inner surface. This leads to a flux equilibrium or a ''stationary membrane reaction'' and explains why the resting potential disappears in the absence of O_2 or presence of HCN (L. de Nó). The electric oscillations of the action potential can be accounted for by another type of chemical reaction, *viz.* a reversible phospho-lipid splitting, which liberates the electrogenic choline or acetylcholine. This reaction, the ''transmembrane reaction,'' sweeps across the membrane; it produces the up-stroke of the ''spike'' when starting at the outer contact surface, and the down-stroke after it has reached the inner contact surface. Definite experimental evidence for this ''trans-membrane reaction'' can be presented.

[1] This investigation was carried out under a grant-in-aid from the National Heart Institute, National Institutes of Health, U. S. Public Health Service.

34. THE RELATION OF THE FLUORESCENCE YIELD OF CHLOROPLASTS TO THE RATE OF THE HILL REACTION. Berger C. Mayne, Rufus Lumry and John D. Spikes, University of Utah.

The fluorescence of chloroplasts isolated from rhubarb, chard and sugar beets *(Beta vulgaris)* was measured using a red-sensitive cesium phototube and a vibrating-capacitor electrometer. Chloroplast fluorescence was produced by focusing the light from a high pressure mercury vapor lamp into a small plastic cell which contained the chloroplast suspension. This light was first passed through a saturated copper sulfate solution and a multilayer interference filter with a transmission

peak at 4390 Å. The light reaching the phototube was passed through two red glass filters to remove the blue light scattered by the chloroplasts. The rate of the photochemical reduction of ferricyanide (Hill reaction) was measured by the potentiometric method in the same cell and optical system used in the measurement of fluorescence.

Fluorescence measurements were made at incident light intensities from those which were approximately saturating for the Hill reaction down to low intensities at which the rate of reaction was approximately a straight line function of intensity. The relative fluorescence yield was found to decrease rapidly as the light intensity approached zero with the maximum curvature of the incident intensity vs. fluorescence yield curve occurring at the intensity which gave one-half the maximum rate of the Hill reaction.

35. AN UNKNOWN PHOSPHATE ESTER ABUNDANT IN PLANTS. N. E. Tolbert, Oak Ridge National Laboratory.

Roots, leaves, and xylem sap from plants labeled with P^{32} and C^{14} have been examined chromatographically. An unknown diester organophosphate with an R_f of 0.9 in phenol-water solvent has been found to be present in all cases. Distribution and tracer experiments indicate that it is synthesized in the root and then transported, apparently by xylem flow, to the leaves where it is utilized. Its rates of accumulation and disappearance are comparable to glutamine and asparagine. In large algal cells that have been ruptured, the unknown phosphate ester is produced rapidly as the cells die. Production of this substance under such conditions indicates the probability that it is formed during old age or seed formation and used up during germination or new growth. Roots of higher plants contain large amounts of this phosphate ester, and as much as 20% of the phosphorus in the exudate of decapitated tomato plants may be present in the compound. In barley seedlings, the organic precursor for this phosphate ester arises from the seed and the phosphorus from the nutrient medium, but in older plants the roots are the sole source of the organic portions. The ester is resistant to acid or base hydrolysis, but phosphate cleaves it to two organic moieties not related to known phospholipids or other phosphate esters in metabolism. Evidence on the identification of these organic fractions will be presented from labeling the unknown with the following C^{14}-labeled compounds: carbon dioxide, formic acid, formaldehyde, glycine, and adenine.

36. KIDNEY FUNCTION IN THE GIANT AFRICAN SNAIL.[1] Arthur W. Martin, Doris M. Stewart and Florence M. Harrison, University of Hawaii and University of Washington.

Morphologically the snail kidney presents no obvious counterparts to the elements of the nephron of the vertebrate. The chief visible microscopic structure is a much-folded membrane of columnar, cuboidal or occasionally stratified epithelial cells. The membrane is bathed on one surface by blood, on the other by urine. Even with so simple a structure it appears that all the processes involved in urine formation in the average vertebrate may be demonstrated in the snail by appropriate methods of study.

By altering the ureteral pressure it has been possible to stop urine formation at a relatively low pressure level, and to demonstrate that the volume of urine flow bears an inverse relationship to the ureteral pressure. Indirect chemical evidence also supports the view that filtration occurs. When an actively secreting kidney is poisoned the excretion of a test agent does not cease but the concentration in the urine falls to that of the blood. This is most easily accounted for on the basis of a continuous filtration process.

Filtration must either be wasteful in salts, water and metabolites or a process of selective reabsorption must be employed. The reabsorption of glucose has been demonstrated as an active process, susceptible to phlorizin poisoning.

Tubule cells of the vertebrate kidney have a well-developed capacity for transporting certain substances against a steep concentration gradient. The epithelial cells of the snail kidney similarly are able to transport such materials as phenol red, paraamino hippuric acid and creatinine against a concentration gradient and this transport may be inhibited by the administration of dinitrophenol.

[1] Supported in part by a contract with the Office of Naval Research.

37. THE APPARENT ADRENERGIC ACTION OF CAFFEINE, THEOPHYLLINE, AND THEOBROMINE ON THE ALLIGATOR. Thomas Hernandez and Roland A. Coulson, Louisiana State University School of Medicine.

Caffeine, theophylline, and theobromine were injected I.P. into alligators in doses of 0.025 to 0.25 gm per kilogram. All three compounds caused a great increase in urine flow and a marked hyperglycemia with glycosuria similar to that found for epinephrine. Following the administration of these xanthines, liver and muscle glycogen are reduced to about 10% of the control level. The fact that the glycogenolytic action of the xanthines exceeds that of epinephrine may be due to their more prolonged action. Caffeine and theophylline also mimic the effects of epinephrine by causing the excretion of an alkaline urine which contains more sodium than chlorine (acid conservation). They affect the central nervous system in much the same fashion as epinephrine, causing pupillary constriction, hyperirritability, and spasticity. The actions of theobromine were much less marked than those of caffeine and theophylline. The results of this study suggest that the action of these xanthines on the alligator is due to an adrenergic effect.

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